Investigating Alternative Methods of Nucleic Acid Delivery in Oocytes

By:
Anita Ramachandran

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Abstract

Studies using RNA interference in oocytes have elucidated processes relating to oocyte function and fertilization. Currently, the only viable method for genetic manipulation of oocytes is microinjection, a very time consuming procedure. There is no quantitative evidence of successful transfections with lipid-based vehicles in oocytes with the zona pellucida intact. Polycations are effective vehicles for delivering nucleic acids into mammalian cells, but have not been tested in oocytes. I investigated whether polycation-mediated nucleic acid delivery is a feasible alternative to microinjection in oocytes. Polyethyleneimine (PEI) is a highly efficient polycationic vector that has been shown to deliver nucleic acids into a variety of cell types, including NIH 3T3 cells. PEI is commonly conjugated to polyethylene glycol (PEG) to reduce cytotoxicity and improve transfection efficiency. Commercial polycation-based nanoparticles, such as HappyFect, are also available for transfections in different cell types. As a functional assay to determine whether the polycations can be used as delivery agents in oocytes, I tested their ability to deliver siRNA that targets a specific gene called *Ensa*. I synthesized PEGylated PEI, characterized it using dynamic light scattering, and examined the ability of unmodified PEI and PEGylated PEI to complex with plasmid DNA or an RNA duplex using gel mobility shift assays. To assess the ability of PEI, PEGylated PEI and HappyFect to knockdown *Ensa* mRNA and ENSA protein in NIH 3T3 cells, I used an *Ensa* specific siRNA cocktail that was previously shown in the Evans Lab to knock down *Ensa* in oocytes via microinjection. Lipofectamine 2000 was used as a positive control, since it was shown to effectively knock down *Ensa* mRNA and ENSA protein in NIH 3T3 cells. Since the

commercial polycation, HappyFect, was able to knock down *Ensa* transcript levels in NIH 3T3 cells, the delivery potential of HappyFect was then explored in oocytes.

Readers/Advisors: Dr. Paul Miller and Dr. Janice Evans

Preface

This thesis is original unpublished work based on research funded by the NIH (R21 HD 069165) conducted at the Johns Hopkins Bloomberg School of Public Health from June 2013 until June 2014. The project was carried out under the supervision of Dr. Paul Miller and Dr. Janice Evans. All relevant literature was accessed through the Welch Library of the Bloomberg School of Public Health portal.

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Introduction

1. Background

1.1. Genetic Manipulation of Oocytes

A greater understanding of biological processes in mammalian oocytes would have a tremendous impact on human reproductive health. In all areas of biomedical research, interfering with the function of gene products or deleting or reducing gene products is a fundamental approach for elucidating mechanisms underlying biological processes. Oocyte biology has benefited significantly from the development of RNA interference. RNAi has been a highly successful method of RNA ablation and subsequent protein knockdown in oocytes (Svoboda & Stein, 2009).

The most commonly utilized method for delivering silencing RNAs (siRNAs) or short hairpin RNAs (shRNAs) into oocytes is microinjection. The microinjection of siRNA or shRNA requires significant skill and expensive equipment. Because microinjection is extremely time consuming and labor-intensive, the development of a time- and cost-effective alternative to microinjection would be highly beneficial for the field of oocyte biology. Such a method to introduce siRNAs or shRNAs would also make it possible to perform large-scale RNAi library screens with mammalian oocytes.

1.2. Methods of Nucleic Acid Delivery

There are multiple methods of delivering nucleic acids into cells. In general, nucleic acid delivery vehicles can be divided into two major categories: viral vectors and synthetic vectors. Each delivery method has specific advantages and disadvantages. Viruses have applications in gene therapy, as they can be transformed into gene-delivery

vehicles by replacing part of the genome of a virus with a therapeutic gene (Pack, Hoffman, Pun, & Stayton, 2005). Synthetic vectors are materials that electrostatically bind DNA or RNA, condense the genetic material into particles a few tens to several hundred nanometers in diameter, and mediate cellular entry of the genetic material (Pack et al., 2005). The majority of synthetic vectors can be further divided into polymers/polycations and lipids. As a result of their relatively high efficiency, lipid based delivery vehicles have been the most widely studied of the synthetic vectors (Zabner, 1997). However, numerous synthetic polycations containing a variety of different chemical compositions have also been employed as vectors for nucleic acid delivery.

1.3. Nucleic Acid Delivery in Oocytes

Ooctyes are female germ cells produced in the ovary during female gametogenesis. The egg coat of the mammalian egg is called the zona pellucida, and is important during fertilization for supporting the binding of sperm. During the growth of an oocyte, a variety of maternally transcribed mRNAs are recruited and translated into proteins that have various functions, from regulation of meiosis and cellular metabolism, to developmental processes like fertilization (Schultz, Letourneau, & Wassarman, 1979; Schultz & Wassarman, 1977). Meiosis in oocytes occurs in a staggered fashion; oocytes are arrested at prophase of meiosis I before birth and the arrest lasts for weeks to years, depending on the species. Upon ovulation, oocytes undergo a transition known as meiotic maturation, during which oocytes exit from prophase I arrest and progress through meiosis I and then arrest at metaphase of meiosis II (Conti, Hsieh, Zamah, & Oh, 2012). Oocytes then complete meiosis II if they are fertilized.

Mouse oocytes and early embryos were the first mammalian tissues where RNAi was documented (Svoboda, Stein, Hayashi, & Schultz, 2000; Wianny & Zernicka-Goetz, 2000). Microinjection is the most commonly utilized delivery method for RNAi in oocytes. Typically, oocytes that are arrested in prophase I are microinjected with shRNAs or siRNAs and then cultured for 24 + hours to allow RNAi-mediated knockdown and protein turnover to occur. Microinjection allows the introduction of siRNA or shRNA into zona pellucida (ZP)-intact oocytes at a defined concentration, and the timing of each experiment can be controlled stringently. While there are many advantages to this method, the technique requires comparatively expensive equipment and demands more time than many of the other methods used for other cell types. Electroporation is another method that has been used for the introduction of siRNA or shRNA into a large number of ZP-intact oocytes and embryos (Grabarek, Plusa, Glover, & Zernicka-Goetz, 2002), but conventional electroporation systems typically require expensive pulse generators (Geng, Zhan, & Lu, 2012).

The use of synthetic vectors would circumvent the need for expensive equipment and reduce the amount of time required for performing RNAi experiments. There are only two reports of successful transfections in mammalian oocytes with lipid-based delivery agents. In one study, Lazar et al. transfected ZP-free rat oocytes that were arrested at prophase I with a dsRNA targeting Cyclin B1 mRNA using a commercial lipid transfection reagent, Lipofectamine (2004). Using RT-PCR, the authors showed that following a 2.5 hour incubation with Lipofectamine and the cyclin B1 dsRNA, cyclin B1 mRNA was not detected in the ZP-free oocytes (Lazar, Gershon, & Dekel, 2004). Additionally, western blot analyses to quantify protein levels, showed that following a

12-hour incubation in the transfection mixture, full ablation of cyclin B1 protein was achieved in the ZP-free oocytes (Lazar et al., 2004).

In another experiment, Carballada et al. transfected ZP-intact mouse oocytes arrested at prophase I with a rhodamine-labeled plasmid DNA using another commercial lipid vehicle, Fugene (2000). The authors determined whether ZP-intact oocytes were successfully transfected with the plasmid by visualizing rhodamine signals. After a 3-hour transfection, they showed that out of 16 oocytes, all were positive for rhodamine labeling (Carballada, Degefa, & Esponda, 2000). As a control, four oocytes were transfected with Fugene alone, and none of these oocytes contained the rhodamine signal (Carballada et al., 2000). However, the choice of transfecting control oocytes with Fugene alone seems questionable because it does not address whether it was the Fugene that delivered the plasmid to the oocytes or whether the rhodamine signals can be seen because of incomplete washes or the incorporation of the plasmid through some other mechanism. A more appropriate control would be treating the oocytes with the plasmid alone. Moreover, using fluorescence microscopy to examine transfection lacks quantitative evidence of the transfection efficiency of these lipid transfection agents in oocytes, and therefore raises questions about the capacity of lipids to transfect ZP-intact oocytes.

Thus, while there is quantitative evidence of using lipid-based delivery vehicles to transfect ZP-free oocytes (Lazar et al., 2004), there is little to no quantitative evidence of using lipids to transfect ZP-intact oocytes. Furthermore, neither report addressed the toxicity of the lipid vectors. Toxicity can be a significant problem with some lipid based delivery agents. Gebhart & Kabanov demonstrated that the toxicity of lipolexes limits the

maximal dose of DNA that can be used in cell transfections; at higher doses of DNA, lipoplexes, made of a variety of commercially available lipid based delivery agents and a plasmid DNA, decreased cellular protein levels to a greater extent than poplypexes, made of a variety of different polycations and the same plasmid DNA (Gebhart & Kabanov, 2001).

In addition to cytotoxicity, lipid-based delivery agents have many other crucial limitations compared to polycations. The formation of lipoplexes involves interactions among lipid molecules, in addition to interactions with the DNA or RNA itself, but the self-assembly of polyplexes does not entail interactions of the polycation molecules with each other (Thomas & Klibanov, 2003). The lipids' hydrophobic segments are the key determinants of the macroscopic characteristics of lipoplexes, such as their size, shape, stability, and interactions with other lipids, cell membranes, and DNA. This, in turn, affects the transfection efficiency of resulting lipoplexes (Smisterova et al., 2001; Zuhorn et al., 2002). While the ability to control these parameters in lipids is rather limited (Simberg et al., 2001), polycations consist of repeating structural units that can be easily manipulated by chemical modifications to achieve higher transfection efficiency.

Another limitation of lipid delivery agents is that lipid based formulations often require adjuvant 'helper lipids' in order to facilitate the fusion of cationic liposomes to the cell membrane (Hui et al., 1996), but polycations are efficient without such adjuvants (Thomas & Klibanov, 2003). Finally, Gebhart and Kabanov also demonstrated that transfection efficiency using a variety of polycations is independent of the presence or absence of serum, while select lipid systems, according to manufacturer's protocols,

should be used in the absence of serum (Gebhart & Kabanov, 2001). This in turn might allow for greater flexibility in some transfection conditions, such as incubation periods.

To date, there is limited evidence of the use of lipid-based delivery agents and no evidence of the use of polycations, or nanoparticles, to transfect oocytes. However, the evidence of transfecting ZP-intact oocytes with lipid-based delivery agents is not quantitative and there is little known about the toxicity of lipid-based delivery agents in oocytes. Furthermore, it has been shown that toxicity is one among many limitations of lipid based delivery agents, as compared with polycations. Both the lack of quantitative evidence of lipid-based transfections in ZP-intact oocytes and the drawbacks of lipid delivery systems as compared with polycations, make polycations a compelling alternative to microinjection for the delivery of nucleic acids into ZP-intact oocytes.

1.4. Polycation Mediated Delivery

Polycations have linear, branched, or dendritic structures, and contain DNA-binding moieties including primary, secondary, tertiary, and quaternary amines, as well as other positively charged groups, such as amidines (Pack et al., 2005). Some commonly used polycations (Figure 1) include polylysine, polyethyleneimine, chitosan, and poly(β-aminoesters).

Delivery of nucleic acids into eukaryotic cells using polycations is a multi-step process that requires condensation of DNA or RNA with the polycation, cellular uptake via endocytosis/pinocytosis, and release from the endosome into the cytosol. Polycations interact electrostatically with the negatively charged phosphates of the DNA or RNA backbone and condense the genetic material into compact, ordered particles called polyplexes. Polyplexes are formulated according to their N/P ratio, which is the ratio of

the moles of nitrogen in the polycation to the moles of phosphate in the nucleic acid. In most cases, the N/P ratio required for polyplex formation is determined empirically. At a certain critical N/P ratio, the nucleic acid undergoes localized bending or distortion that allows the formation polyplexes, which are typically toroidal or spherical structures (Hansma et al., 1998; Wagner, Cotten, Foisner, & Birnstiel, 1991) and range from 20-200 nm in diameter (Dunlap, Maggi, Soria, & Monaco, 1997; Golan, Pietrasanta, Hsieh, & Hansma, 1999; Lynn, Anderson, Putnam, & Langer, 2001)

Once formed, polyplexes can bind electrostatically to the surface of cells and are internalized via adsorptive pinocytosis (Mislick & Baldeschwieler, 1996). Alternatively, if the polyplexes contain targeting ligands, they can bind to specific cell-surface receptors and are internalized by receptor-mediated endocytosis. In either case, the polyplexes are localized within endocytic vesicles inside the cells. The first vesicle in which the polyplex is localized fuses with sorting endosomes from which the material can either be transported out of the cell by exocytosis, or into late endosomes, vesicles that are acidic (pH 5-6) due to ATPase proton pumps in the vesicle membrane. Polyplexes are then trafficked into lysosomes that contain various degradative enzymes. In order to deliver their nucleic acid cargo, polyplexes contained in the late endosomes must be able to escape into the cytoplasm before being degraded by endolysosomes.

Several strategies have been developed to ensure that polyplexes are able to escape from late endosomes and evade lysosomal degradation. Treating cells at the time of transfection with chloroquine, which buffers the pH of acidic vesicles, has been shown to promote endosomal escape and therefore improve transfection efficiency of some polymers (Erbacher, Roche, Monsigny, & Midoux, 1996). Additionally, fusogenic

synthetic peptides, which undergo structural changes at acidic pH and disrupt vesicle membranes, have been attached to polymers in order to promote endosomal escape (Lee, Jeong, & Park, 2001; Vaysse, Burgelin, Merlio, & Arveiler, 2000; Wyman et al., 1997). Finally, some polymers, such as polyethyleneimine (PEI) and polyamidoamine (PAMAM) dendrimers, are thought to mediate their own endosomal escape through a mechanism known as the 'proton sponge effect' (Boussif et al., 1995).

1.5. Polyethyleneimine and the Proton Sponge Effect

Polyethyleneimine is one of the most effective gene-delivery polymers and has been shown to efficiently deliver nucleic acids into a variety of different mammalian cells in culture (Boussif et al., 1995). The relatively high nucleic acid delivery efficiency of PEI is believed to be due primarily to the efficient escape of polyplexes from the endocytic pathway. PEI is an organic macromolecule that can exist in a linear or branched form. It is available in a broad range of molecular weights. The molecular weight of PEI most suitable for gene transfer ranges between 5 and 25 kD (Neu, Fischer, & Kissel, 2005). Higher molecular weight PEI tends to be more cytotoxic (Fischer, Li, Ahlemeyer, Krieglstein, & Kissel, 2003) due to aggregation of clusters of the polymer that induces necrosis (Fischer, Bieber, Li, Elsasser, & Kissel, 1999).

Because PEI is rich in secondary and tertiary amines, it is readily protonated in the acidic environment of the endosome, and consequently has a very high cationic charge density potential (Boussif et al., 1995). Thus, PEI polyplexes act as proton sponges inside the endosome, resulting in an influx of protons that stimulates the entrance of water from the cytosol to balance the high osmotic pressure. The subsequent swelling

eventually leads to the rupture of the endosome and release of the PEI polyplexes into the cytosol.

PEI has been used as a delivery vehicle in a variety of different mammalian cell lines, including NIH 3T3 murine fibroblasts, COS-7 monkey kidney, HepG2 human hepatoma, HeLa human epithelial carcinoma, and many more (Boussif et al., 1995). Although PEI-mediated transfections have been shown to be particularly efficient as compared to other polycations, such as lipopolyamine and polylysine (Boussif et al., 1995), PEI polyplexes can be toxic to cells. This toxicity can be significantly reduced by conjugating PEI with polyethylene glycol (PEG). PEGylation of PEI shields the surface charges of PEI and prevents the polycation from interacting with other negatively charged macromolecules inside the cell; this in turn reduces cytotoxicity (Petersen et al., 2002). In addition, PEGylation improves transfection efficiency by weakening electrostatic interactions between PEI and its nucleic acid cargo, which in turn allows the nucleic acid to be easily released into the cytoplasm (Mao et al., 2006).

1.6. Endocytosis in Oocytes

Polycations are taken up into cells via endocytosis. Therefore, the ability of oocytes to undergo endocytosis is crucial for polycation-mediated delivery to be a feasible alternative to microinjection. Oocytes do in fact undergo endocytosis. Lowther et al. showed that dynamin and clathrin, two of the most common proteins responsible for receptor endocytosis in somatic cells, are expressed in oocytes (2011). They also demonstrated that when oocytes were incubated in FM-143, a dye that incorporates into the plasma membrane of cells and retains its fluorescence when the membrane is endocytosed, fluorescence could be detected in endosomes in the cytoplasm of the

oocytes (Lowther, Nikolaev, & Mehlmann, 2011). It has also been shown that receptor-mediated endocytosis is crucial for some oocyte biological processes, particularly the proper regulation of cAMP levels that is required for the maintenance of meiotic arrest (Lowther et al., 2011). cAMP is produced in oocytes by the activity of a constitutively active G-protein-coupled receptor 3 (GPR3) and it has been shown that this GPR3 localizes in the plasma membrane and within early endosomes in the oocyte, which indicates that GPR3 is endocytosed (Lowther et al., 2011). Furthermore, inhibition of endocytosis disrupts the proper regulation of cAMP levels (Lowther et al., 2011).

2. Thesis Aims and Objectives

This thesis investigates whether the delivery of nucleic acids into mammalian oocytes using polycations may be a potential alternative to microinjection. Lipid-based delivery vehicles have been shown in a single study to deliver dsRNA into ZP-free oocytes, but it is unclear how effective these vehicles are in transfecting ZP-intact oocytes. Moreover, studies examining polycation-mediated delivery in a variety of other cell types have shown that polycation-based delivery systems are potentially superior to lipid-based systems in many respects. Thus, I sought to examine the potential of polycations to deliver nucleic acids into ZP-intact oocytes. In particular, I aimed to test the delivery potential of three different types of polycations: PEI, PEGylated PEI, and a commercial polycation-based nanoparticle, HappyFect. Unmodified PEI was purchased from a manufacturer; PEGylated PEI was made by covalently conjugating PEG to PEI. I then used dynamic light scattering and gel mobility shift assays to physically and chemically characterize the unmodified PEI and PEGylated PEI based on their size and their ability to complex with DNA or RNA.

Because PEI and PEG-PEI have been shown to effectively deliver nucleic acids into mouse NIH 3T3 cells (Boussif et al., 1995; Petersen et al., 2002), I first tested the delivery potential of the three polycations in NIH 3T3 cells before testing them in mouse oocytes. To test the delivery potential of the polycations in NIH 3T3 cells, I used the polycations as vehicles to deliver siRNAs that target a specific gene, and then examined knockdown of the gene at the transcript or protein level. To do this I targeted *Ensa* with an siRNA cocktail that had been shown previously by the Evans lab to knock down expression of *Ensa* mRNA and ENSA protein in oocytes using the gold standard delivery method of microinjection (Matthews and Evans, 2014).

To ensure that the *Ensa*-targeting siRNA was functional in NIH 3T3 cells, I first used Lipofectamine 2000, a lipid-based delivery vehicle, to deliver the siRNA. I showed that the *Ensa* siRNA did achieve knockdown in the NIH 3T3 cells at both the transcript and protein level using qPCR and western blots to determine the levels of *Ensa* mRNA and protein respectively in cells transfected with Lipofectamine 2000 and *Ensa* siRNA. I then examined the ability of PEI, PEG-PEI, and HappyFect to deliver the *Ensa* siRNA and knock down *Ensa* mRNA and protein. These experiments were conducted with Lipofectamine 2000 as a positive control delivery vehicle. Because HappyFect was the only polycation to achieve significant knockdown of *Ensa* mRNA and protein in NIH 3T3 cells, I then tested the ability of HappyFect to deliver *Ensa* siRNA in mouse oocytes.

Materials and Methods

1. Synthesis and Physico-chemical Characterization of Polycations

1.1. Synthesis and Physical Characterization of PEG-PEI

Synthesis

PEGylated PEI, containing 25 kD branched polyethylene-imine conjugated to a maleimide amine derivative of a 3.4 kD polyethylene glycol was synthesized according to the schematic shown in Figure 2 using a protocol from Chen et al. (2012). PEI (Sigma-Aldrich; 125 mg) was dissolved in 50 mL of 0.1 M HEPES buffer (pH 8.4) and stirred overnight. N-succinimidyl-S-acetylthiopropionate (SATP, Thermo Scientific; 12 mg) was dissolved in 8 mL of anhydrous DMSO, which was prepared by drying DMSO using 3-angstrom molecular sieves. SATP (4.8 μmoles or 1.2 mg) dissolved in 800 μL of DMSO was slowly added to 4 mL of the PEI solution (0.4 μmol or 10 mg). The mixture was stirred at room temperature for 2 hours, and the resulting product was purified using a 15 mL 10 kD molecular-weight cut-off centrifuge filtration tube (Amicon). All centrifugations described in this section occurred at 4000 x g for 40 minutes. After centrifuging once, 400 μL of 1X PBS was added to the centrifuge tube and centrifuged again. This was repeated 3-4 times until 200 μL of the concentrate was left. When PEI reacts with SATP, an acetylated PEI molecule containing a protected sulfhydryl group is formed.

The resulting product was deacetylated by treatment with hydroxylamine-hydrochloric acid. A 400 μL aliquot of deacetylating solution, containing 0.5 M hydroxylamine-HCl and 25 mM in EDTA in 1X PBS (pH 7.2-7.5), was slowly added to

the acetylated PEI from the first step of the synthesis and stirred at room temperature for 2 hours. The solution was transferred to a 15 mL MW cut-off centrifuge tube and centrifuged. After one spin, 400 μL of 0.1 M HEPES buffer was added to the centrifuge tube and centrifugation was repeated. This process was repeated 3-4 times until 200 μL of the concentrate was left. The resulting product is a PEI molecule with a free reactive thiol group.

The free thiol group of PEI was reacted with PEG that was derivatized with a maleimide. Maleimide PEG amine (NANOCS, 13.6 mg or 4μmoles) dissolved in 1.6 mL of anhydrous DMSO was slowly added to the PEI molecule from Step 2. The reaction mixture was transferred to a MW cut-off centrifuge tube. After one spin, 1 mL of water was added and centrifugation was repeated. This process was repeated 3-4 times. The resulting product was analyzed using dynamic light scattering, lyophilized on a Speed Vac, and dissolved in water. The concentration of the PEG-PEI solution was determined by weighing the lyophilized product before dissolving in water. The molar ratio of PEI to PEG was 1:10. The expected molecular weight is 59 kD assuming that all the PEG reacts with the PEI.

Characterization of PEG-PEI by Dynamic Light Scattering

Dynamic light scattering was used to characterize the size of PEG-PEI by comparing the average hydrodynamic radius of particles in a solution of PEI alone to that of a solution of PEG-PEI. PEI or PEG-PEI was dissolved in a HEPES Buffered Saline (HBS) solution that contained 20 mM HEPES and 150 mM NaCl. The concentration of the PEI solution was 1mg/mL. The concentration of the PEG-PEI solution was unknown as the hydrodynamic diameter was measured before lyophilizing the final product. Five

aliquots (50 μL) of each solution were measured on a DynaPro instrument with DYNAMICS software using regularization analysis. Each measurement consisted of 20 acquisitions, 10 seconds each. The average hydrodynamic radius of 5 measurements as well as the standard errors of the measurements were determined, in order to examine variation between aliquots of each solution.

2. Chemical Characterization of Polycations: Polyplex Formation

2.1 Calculating N/P Ratios

All experiments involving the formation of polyplexes require calculations of the N/P ratio, the ratio of the moles of nitrogen in PEI or PEG-PEI to the moles of phosphate in the nucleic acid. As in convention in the field, the N/P ratio will be expressed as one number and an N/P ratio of 10, for example, will signify ten moles of nitrogen for every one mole of phosphate (Boussif et al., 1995; Petersen et al., 2002; Zhao et al., 2009). In each experiment, the amount of nucleic acid was kept constant but the amount of PEI or PEG-PEI varied to give different N/P ratios. The moles of phosphate were calculated using either the weight of the nucleic acid or the number of base pairs. In the experiments using plasmid DNA, 20 ng of plasmid were used to form each complex. The average molecular weight of a nucleotide is 330g/mol. Thus, 20 ng of plasmid is equivalent to 61 pmoles of phosphate (20×10^{-9} g / 330 g/mol). In the experiments using the RNA duplex that mimics the Ensa siRNA or the *Ensa*-targeting siRNA, the number of base pairs was used to calculate the moles of phosphate. Both the duplex and the siRNA contained 19-bp or 36 phosphates per mole of the duplex. Thus, 10 pmoles of the duplex is equivalent to 360 pmoles of phosphate (10×10^{-12} moles * 36 phosphates/mol).

The moles of nitrogen required to form the complex were calculated based on the moles of phosphate multiplied by the N/P ratio. For example, if the desired N/P ratio is 10 and the complex contains 360 pmoles of phosphate, we would need 3.6 nmoles of nitrogen

Branched 25 kD polyethyleneimine is made up of *n* repeating units of a monomer, $C_{22}H_{55}N_{11,}$ whose molecular weight is 473 D. There are a total of 53 (25,000/473) monomer units each containing 11 nitrogens and thus a total of 583 (53 x 11) moles of nitrogen in each mole of PEI or PEG-PEI. Therefore at an N/P ratio of 10, 617 pmoles (3.6×10^{-9} nitrogens/583 moles of nitrogen/mol) of PEI or PEG-PEI are required to form the complex.

2.2. Complex Formation with a Plasmid

pGEM-3Z (165 ng) was treated with 2 μL of restriction enzyme EcoRI in a solution of 5 μL of EcoRI buffer and 37 μL of water. The reaction was incubated for 2 hours at 37 °C. Five aliquots (for 5 complexes) of 20 ng of the linearized plasmid was evaporated under vacuum and residue was dissolved in 5 μL of HEPES Buffered Saline (HBS) solution. PEI or PEG-PEI, dissolved in 5 μL of HBS, was added to the 20 ng aliquots of the pGEM-3Z at N/P ratios of 1, 5, 10, 15 and 20 (See Table 1 for molar amounts of polymers); total volume of each solution was 10 μL. The solutions were incubated at room temperature for 20 minutes and loaded onto a 0.8% TAE agarose gel (8.3 cm x 5 cm) containing SYBR Green. The gel was run in TAE buffer at 100V for 1 hour, and the complexes were visualized in the gel using a phosphorimager.

2.2 Complex Formation with an RNA Duplex

The duplex, which mimicked the Ensa-targeting siRNA, contained a FITC labeled oligo-2'-O-methylribonucleotide annealed to a complementary RNA. Five pmoles of the duplex were used for each complex with PEI or PEG-PEI; a stock solution of duplex was made by combining equivalent amounts (300 pmoles) of the FITC-oligo and its complementary RNA in 300 μL of HBS; the duplex was heated at 95 °C for 5 minutes and allowed to slow cool to room temperature for annealing of the strands to occur. To confirm duplex formation, 5 μL of the FITC-oligo and the duplex were loaded onto a 20% native polyacrylamide gel (8 cm x 8 cm) and run in TBE buffer at 100 V. Fluorescence was visualized with a phosphorimager.

PEI or PEG-PEI, dissolved in 5 μL of HBS, was added to 5 μL (5 pmoles) of the duplex in HBS at N/P ratios of 5 to 40 at increments of 5 (See Table 2 for molar amounts of polycations). The solutions were incubated at room temperature for 20 minutes and loaded onto a 1% TBE agarose gel (8.3 cm x 5 cm) and the gel was run in TBE buffer at 100V for 30 minutes. A phosphorimager was used to visualize the fluorescence of the FITC labeled duplex.

3. siRNA Mediated Knockdown of Ensa in NIH 3T3 cells

3.1 Cell Culture

For each transfection experiment, mouse NIH 3T3 fibroblast cells were seeded in 6-well plates at a density of 1 x 10^5 cells/mL in 2-2.5 mL of Dulbecco's modified Eagle's medium (DMEM, Gibco by Life Technologies) supplemented with 10% fetal bovine

serum (FBS, Gibco by Life Technologies), hereafter referred to as serum-complete DMEM, and incubated at 37 °C in a 5% CO_2 atmosphere overnight. Cells were at 50-80% confluence for transfection experiments. Transcript level transfection experiments were performed in triplicates (three wells of siRNA-treated cells and three wells of control cells); protein level transcription experiments were conducted with 1 well of siRNA-treated cells and 1 well of control cells.

3.2. Preparing the *Ensa*-targeting siRNA

ON-TARGETplus SMARTpool siRNA (Thermo Scientific) targeting mouse *Ensa* (see Table 3 for sequences; used previously for RNAi-mediated knockdown of *Ensa* in mouse oocytes (Matthews and Evans, 2014)) was dissolved according to manufacturer's instructions, to a concentration of 100 µM in four volumes of RNase-free water and one volume of 5X siRNA Buffer. For the transfections, the100 µM siRNA stocks were diluted to a 20 µM solution in 1X siRNA buffer.

3.3. Transfections

Lipofectamine

For all transfections using Lipofectamine 2000 as a delivery vehicle, 3 µL of Lipofectamine 2000 diluted in 100 µL of OptiMEM (Gibco by Life Technologies) were added to 100 pmoles of the *Ensa* siRNA diluted in 100 µL of OptiMEM, for a total transfection mixture volume of 200 µL. For control cells, 3 µL of Lipofectamine 2000 were diluted in 200 µL of OptiMEM without the siRNA. Solutions were mixed by pipetting up and down and incubated at room temperature for 20 minutes. Solutions were then added dropwise to cells in 1.8 mL of serum free DMEM (total volume of 2 mL). After 4 hours of incubation in 37 °C/5% CO_2, the medium was replaced with serum-

complete DMEM and the cells were cultured for 44-48 hours before the isolation of RNA or western blot analysis.

PEI, and PEG-PEI

N/P Ratio of 10

Transfection conditions were adapted from Chen et al. (2012). PEI or PEG-PEI (62 pmol) diluted in 100 µL of 1X PBS were added to 100 pmoles of the *Ensa* siRNA diluted in 100 µL of 1X PBS, for a total transfection mixture volume of 200 µL. For control cells, 62 pmoles of PEI or PEG-PEI were diluted in 200 µL of 1X PBS without the siRNA. Solutions were mixed by pipetting up and down and incubated at room temperature for 20 minutes. Solutions were then added dropwise to cells in 1.8 mL of serum-complete DMEM (total volume of 2 mL). After 4 hours of incubation in 37 °C/5% CO_2, the medium was replaced with fresh serum-complete DMEM and the cells were cultured for 44-48 hours before the isolation of RNA.

N/P Ratio of 20

Transfection protocol was modified to keep the conditions of complex formation the same as those used for the gel mobility shift assays with the RNA duplex. PEI or PEG-PEI (124 pmol) diluted in 100 µL of HBS were added to 100 pmoles of the *Ensa* siRNA diluted in 100 µL of HBS, for a total transfection mixture volume of 200 µL. For control cells, 124 pmoles of PEI or PEG-PEI were diluted in 200 µL of HBS without the siRNA. Solutions were mixed by pipetting up and down, and incubated at room temperature for 20 minutes. Solutions were then added dropwise to cells in 1.8 mL of serum-free DMEM (total volume of 2 mL). After 4 hours of incubation in 37 °C/5% CO_2,

the medium was replaced with fresh serum-complete DMEM and the cells were cultured for 44-48 hours before the isolation of RNA.

HappyFect

HappyFect was incubated in 37 °C for 10 minutes prior to use, vortexed and centrifuged quickly. According to manufacturer's instructions, the suggested amounts of HappyFect (Table 4) can be varied +/- 50%. Two separate experiments were performed in which two different amounts were tested: 10 µL in a total volume of 2.5 mL of DMEM (0.4% HappyFect) or 20 µL in a total volume of 2.5 mL of DMEM (0.8% HappyFect). In each experiment, 10 or 20 µL of HappyFect, diluted in 62.5 µL of OptiMEM were added to 100 pmoles of the *Ensa* siRNA, diluted in 62.5 µL of OptiMEM, for a total transfection mixture volume of 125 µL. For the control cells, 10 or 20 µL of HappyFect were dissolved in 125 µL of OptiMEM without the siRNA. Solutions were incubated at room temperature for 20 minutes and 375 µL of serum-free media was added to the solutions. The media containing the transfection solutions was then added to the cells along with an additional 2 mL of fresh serum-free media (for a total volume of 2.5 mL). After 4 hours of incubation in 37 °C/5% CO_2, media was replaced with serum-complete DMEM and cells were cultured for 44-48 hours before the isolation of RNA and western blot analysis.

3.4. Evaluating Knockdown at the Transcript Level

RNA Extraction/cDNA Synthesis

RNA was isolated from NIH 3T3 cells using the 5 PRIME PerfectPure RNA Cultured Cell Kit. PerfectPure Lysis Solution (400 µL) was added directly to each well of cells. Cells were scraped and the cell lysate was pippetted up and down for thorough homogenization. Cell lysate was then added to a purification column where RNA was bound to a membrane and washed with buffers provided in the kit. During the purification, the column was treated with DNase for 15 minutes according to manufacturer's instructions. RNA was eluted in an elution buffer containing DEPC-treated water and RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). The total volume of each cDNA reaction was 20 µL, which contained 0.5 -1 µg of RNA, 4 uL of the 5X iScript buffer, 1µL of iScript reverse transcriptase, and nuclease free water.

RT-qPCR

Quantitative PCR was performed using primers specific to mouse *Ensa* and a reference gene, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (See Table 5 for primer sequences). To calculate primer efficiencies, standard curves were made by preparing reactions with serial dilutions of the template, NIH 3T3 cDNA. All qPCR reactions were prepared using 10 µL of SsoAdvanced SYBR Green Supermix (Bio-Rad), 1.5 µL of a 5 µM primer solution containing forward and reverse *Ensa* or *Gapdh* primers, 3.5 µL of nuclease-free water, and 5 µL of template, for a total volume of 20 µL. *Ensa* and *Gapdh* mRNA levels were detected with a CFX96 Real-Time PCR Detection

Instrument using the following cycle conditions: 95°C for 2 minutes, followed by 35 cycles of 95°C for 15 seconds, 55°C for 20 seconds and 72°C for 30 seconds. Cycle Threshold (Ct) values of all samples were recorded, and fold changes ($\Delta\Delta$Ct) were calculated by normalizing *Ensa* expression against *Gapdh* expression.

3.5 Evaluating Knockdown at the Protein Level

Prior to western blot analysis, cells were suspended in 300 uL of SDS-PAGE sample buffer (3% SDS, 10% glycerol, 0.02% bromophenol blue, 4% 2-mercaptoethanol, 65 mM TRIS-HCl, pH 6.8) and cell lysate was sonicated. Samples were then boiled at 100 °C for 10 minutes and various amounts of the control cell lysate and the *Ensa* siRNA lysate were loaded onto a 12.5% polyacrylamide SDS-gel to separate the proteins.

Western Blot for Lipofectamine Transfections

Lipofectamine knockdown experiments were performed in collaboration with Lauren Matthews; I performed cell culture work, transfection, RNA extraction, cDNA synthesis, and RT-qPCR (described above); LM performed protein extraction and immunoblotting. Two separate gels were run for each antibody (ENSA and Actin) with different amounts of cell lysate because the Actin signal was saturated when the minimum amount of cell lysate required for the ENSA signal was loaded onto the gel. Thus, to stay within linear range of detection, for the anti-ENSA blot, 0.5, 1, 2, and 5 µL of cell lysate was loaded onto the gel and for the anti-Actin blot, 0.025, 0.05, 0.1, and 0.25 µL of cell lysate was loaded onto the gel. The proteins were transferred to two Immobilon-P PVDF membranes (Millipore) and each membrane was probed with Actin (50kD) and ENSA (25 kD) separately.

Actin

The membrane was blocked overnight in 5% dry milk in PBS-T (PBS containing 0.1% Tween-20 (Sigma-Aldrich). The membrane was then incubated with 10 mL of an anti-ACTIN antibody (Santa Cruz Biotechnology; made against full-length mouse Actin; diluted 1:20,000 in PBS-T + 5% BSA + 0.02% NaN_3) for 1 hour, washed 3 times with PBS-T, and then incubated with a goat anti-mouse IgG-horseradish peroxidase (HRP) (Thermo Scientific; diluted 1:20,000 in PBS-T + 3% BSA) for 1 hour, followed by 3 washes with PBS-T.

ENSA

The membrane was blocked overnight in 10% cold-water fish gelatin in PBS-T. The membrane was then incubated with an anti-ENSA antibody (Santa Cruz Biotechnology; made against full-length human ENSA; diluted 1:400 in PBS-T containing 3% BSA and 0.012% $NaN_{3)}$ for 2 hours, washed 3 times with PBS-T, and then incubated with a goat anti-rabbit IgG-horseradish peroxidase (HRP) (Jackson Immunoresearch; diluted 1:2000 in PBS-T plus 3% BSA) for 1.5 hours, followed by 3 washes with PBS-T.

Protein Detection

The membranes were incubated in Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) for 5 minutes and exposed to X-ray film. X-ray film was scanned using an HP LaserJet 3390 scanner and ImageJ software was used to analyze band intensity. ImageJ software was used to analyze band intensity. The rectangular selection tool was used to select each band, and peak intensity was determined. The area

under each peak was calculated as a measure of band intensity. To calculate knockdown, the ENSA bands of 0.5 µL of cell lysate were normalized to the Actin bands of 0.1 µL of cell lysate.

Western Blot for HappyFect Transfections

One gel was loaded with 1, 5, 10, and 15 µL of cell lysate. Proteins were transferred to a 0.45 µm nitrocellulose membrane (BioRad). After the transfer, the membrane was cut at the 31 kD line of the ladder to probe for GAPDH (37 kD) and ENSA (25 kD) separately. The ENSA blotting protocol is the same as that used for the western blots for the Lipofectamine transfections.

GAPDH

The membrane was blocked overnight in 5% dry milk in PBS-T (PBS containing 0.1% Tween-20 (Sigma-Aldrich). The membrane was then incubated with an anti-GAPDH antibody (Santa Cruz Biotechnology; made against full-length mouse GAPDH; diluted 1:5000 in blocking solution) for 1 hour, washed 3 times with PBS-T, and then incubated with a goat anti-mouse IgG-horseradish peroxidase (HRP) (Thermo Scientific; diluted 1:5000 in the blocking solution) for 1 hour, followed by 3 washes with PBS-T.

Protein Detection

Membranes were incubated in Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) for approximately 5 minutes and scanned using a FluorChem Q imaging system. ImageJ software was used to analyze band intensity, as described for the Lipofectamine blot. To calculate knockdown, the intensities of the ENSA bands were

normalized to the intensities of the GAPDH bands obtained with the same amount cell lysate and the knockdown percentages were averaged.

4. Uptake of the FITC-Duplex using PEI as a delivery Vehicle

4.1 Cell Culture

Mouse NIH 3T3 fibroblast cells were seeded in glass bottom culture dishes at a density of 2 x 10^5 cells/mL in 2 mL of DMEM with FBS and incubated at 37 °C and a 5% CO_2 atmosphere overnight. Cells were at 50-80% confluence before transfection experiments were performed.

4.2. Transfection

PEI or PEG-PEI (741 pmol), diluted in 250 µL of HBS, was added to 600 pmoles of the FITC-duplex diluted in 250 µL of HBS, for a total transfection mixture volume of 500 µL (N/P ratio of 20). Lipofectamine was used as a positive control. Lipofectamine 2000 (12 µL) was diluted in 250 µL of OptiMEM and added to 600 pmoles of the FITC-duplex diluted in 250 µL of OptiMEM, for a total transfection mixture volume of 500 µL. Solutions were mixed by pipetting up and down and incubated at room temperature for 20 minutes. Solutions were then added dropwise to cells in 1.5 mL of serum-free DMEM (total volume of 2 mL). After 4 hours of incubation in 37 °C/5% CO_2, the medium was replaced with serum complete DMEM. Following a culture period of 24 hours, cells were examined under a microscope.

4.3. Live Cell Microscopy

Microscopic imaging was performed on a Zeiss Axio Observer Z1 microscope and AxioVision software. Images were taken with 10X resolution using differential interference contrast (DIC).

5. Treatment of oocytes with HappyFect to introduce *Ensa*-targeting siRNA

5.1 Oocyte Collection and Culture

Animals were used in accordance with the guidelines of the Johns Hopkins University Animal Care and Use committee. Germinal vesicle-intact (GVI) (prophase I-arrested) oocytes were collected from 6-8-week-old female CF-1 mice (Harlan, Indianapolis, IN) in Whitten's-HEPES medium (109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid hemicalcium salt (Whitten, 1971), supplemented with 7 mM $NaHCO_3$ and 15 mM HEPES). Dibutyryl cAMP (dbCAMP, 0.25 mM) was added to culture medium to maintain prophase I arrest (Cho, Stern, & Biggers, 1974). Ovaries were punctured with syringe needles to release oocyte-cumulus complexes from ovarian follicles, and cumulus cells were removed from the oocytes by pipetting oocyte-cumulus complexes through a thin-bore pipette. Oocytes were then transferred to Whitten's-Bicarbonate medium containing 0.05% PVA and 0.25 mM dbcAMP for culture (hereafter referred to as WB medium), and allowed to recover for 1 hour in 37 °C and a 5% CO_2 atmosphere.

5.2 Transfection using HappyFect

Oocytes were treated with HappyFect, following the manufacturer's suggested protocol for 10 pmoles of siRNA (Table 4). Since the amounts of HappyFect could be varied +/- 50%, as a preliminary experiment, 25 oocytes were transferred to a 35 µL drop of a solution containing 1µL of HappyFect in 500 µL of WB medium (0.2% HappyFect) and 25 oocytes were transferred to a 35 µL drop of a solution containing 2µL of HappyFect in 500 µL of WB medium (0.4% HappyFect).

As in the experiments with NIH 3T3 cells, prior to the transfection, HappyFect was incubated in 37 °C for 10 minutes, vortexed and centrifuged quickly. During the transfection, 1 µL of HappyFect was added to 10 pmoles of the *Ensa* siRNA in 25 µL of OptiMEM. For the control cells, 1 µL of HappyFect was added to 10 pmoles of a negative control siRNA (On-TARGETplus control pool (Dharmacon)) in 25 µL of OptiMEM. Solutions were incubated at room temperature for 20 minutes and then diluted in 500 µL of WB medium. Sixty oocytes were transferred to a 35 µL drop of the solution containing HappyFect and the *Ensa* siRNA and 60 oocytes were transferred to a 35 µL drop of the solution containing HappyFect and the negative control siRNA. Additionally, as a visual control for toxicity, 20 oocytes were transferred to a drop containing only WB medium. After 4 hours of incubation in 37 °C/5% CO_2, oocytes treated with HappyFect were washed by transferring to 5 different drops of WB media. Finally, all oocytes were transferred to a fresh drop of WB medium and cultured in 37 °C/5 % CO_2 for 44-48 hours.

5.3. Evaluating Knockdown at the Protein Level

Western Blots

Prior to western blot analysis, 20 oocytes from each experimental group were suspended in 10 µL of SDS-PAGE sample buffer (3% SDS, 10% glycerol, 0.02% bromophenol blue, 4% 2-mercaptoethanol, 65 mM TRIS-HCl, pH 6.8). Samples were boiled at 100 °C for 10 minutes and loaded onto a 12.5% polyacrylamide SDS-gel to separate the proteins. Proteins were transferred to an Immobilon-P PVDF membrane. After the transfer, the membrane was cut at the 31 kD line of the ladder to probe for GAPDH (37 kD) and ENSA (25 kD) separately. The blotting protocol using the anti-ENSA was the same as that used for the NIH-3T3 cell lysates. For the anti-GAPDH blot, the membrane was blocked overnight in 5% dry milk in PBS-T, incubated with an anti-GAPDH antibody diluted 1:3000 in PBS-T + 3% BSA) for 1 hour, washed 3 times with PBS-T, and then incubated with a goat anti-mouse IgG-horseradish peroxidase (HRP) diluted 1:3000 in PBS-T + 3% BSA) for 1 hour, followed by 3 washes with PBS-T.

Protein Detection

The membrane was incubated in Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) for 5 minutes and exposed to X-ray film. X-ray film was scanned using an HP LaserJet 3390 scanner and ImageJ software was used to analyze band intensity.

Results

1. Synthesis and Physico-chemical Characterization of Polycations

1.1. Synthesis and Physical Characterization of PEG-PEI

After synthesizing PEGylated PEI, containing 25 kD branched polyethylene-imine conjugated to a maleimide amine derivative of a 3.4 kD polyethylene glycol, the size of unmodified PEI and PEGylated PEI was characterized using dynamic light scattering, based on the fact that PEGylated PEI is expected to have a larger molecular weight than PEI alone. As determined by five different measurements of PEI or PEG-PEI solutions, the average hydrodynamic radius of PEI was smaller than that of PEG-PEI, consistent with the formation of PEG conjugates on PEI (Figure 3).

2. Chemical Characterization of Polycations: Polyplex Formation

2.1. Complex Formation with a Plasmid

To assess the ability of PEI, and PEG-PEI to complex with nucleic acids, I first determined whether the polycations could complex with plasmid DNA, using gel mobility shift assays adapted from Zhang et al. (2004). Linearized pGEM-3Z plasmid and PEI or PEGylated PEI were combined at different N/P ratios, and loaded onto an agarose gel. The agarose gels contained SYBR green, a dye that binds to DNA; complete complexation occurs when SYBR green is excluded from the complex and is unable to bind to the plasmid, resulting in the disappearance of the dye on the gel. When PEGylated PEI or PEI was added to the linearized pGEM plasmid and loaded onto a gel,

PEI formed a complex with the plasmid at N/P ratios ≥10, while PEG-PEI formed a complex with the plasmid at N/P ratios ≥ 5 (Figure 4).

2.2 Complex Formation with a Duplex

Because I used siRNA mediated knockdown as a functional assay to examine whether PEI or PEG-PEI would deliver nucleic acids into cells, it was important to investigate whether the polymers could complex with siRNA in addition to plasmids. Moreover, due to the possibility that the polymers do not form complexes with siRNA at the same N/P ratios that they form complexes with plasmid DNA, gel mobility shift experiments adapted from Li et al. (2010) were conducted with a duplex that mimics the Ensa siRNA. The duplex contained a FITC labeled oligo-2'-O-methylribonucleotide (19 nucleotides in length) annealed to a complementary RNA. First, to assess duplex formation, the FITC-oligo and the duplex were loaded onto a native polyacrylamide gel (Figure 5A). The shift in mobility of the duplex as compared with the FITC-oligo confirmed duplex formation.

PEGylated PEI or PEI was added to the FITC labeled duplex at varying N/P ratios, and complexes were loaded onto an agarose gel as shown in Figure 5B. Quenching of the FITC fluorescence indicates polyplex formation (Li et al., 2010). Although PEI formed a complex with the duplex at N/P ratios ≥ 20, it is unclear if the decrease in fluorescence intensity seen with PEG-PEI is due to complex formation.

3. siRNA Mediated Knockdown of *Ensa* in NIH 3T3 cells

Recent work in the Evans lab demonstrated that the protein α-endosulfine (ENSA) plays a role in the exit of oocytes from prophase I arrest (Matthews and Evans, 2014). These studies utilized oocytes microinjected with negative control siRNA or with *Ensa*-targeting siRNA; the injected oocytes were then cultured in conditions to maintain prophase I arrest to allow time for knockdown of the target *Ensa* mRNA to occur (Matthews & Evans, 2014). Following a 44-48 hour culture period, *Ensa* mRNA levels decreased by 67 ± 11% and ENSA protein levels decreased by 80 ± 6% in *Ensa* siRNA-injected oocytes as compared with controls (Matthews & Evans, 2014). Before examining the ability of the polycations to deliver the *Ensa* siRNA, I needed to make sure the siRNA cocktail functions in NIH 3T3 cells. To assess the ability of the *Ensa* siRNA to effectively knock down *Ensa* expression in NIH 3T3 cells, I first used Lipofectamine 2000 as a delivery vehicle because it is known to deliver DNA or RNA into this cell type (Elbashir et al., 2001).

Use of Lipofectamine to Introduce the Ensa-targeting siRNA

Triplicates of NIH 3T3 cells were transfected with Lipofectamine 2000 (control) or a complex consisting of Lipofectamine 2000 and the *Ensa* siRNA. Following a culture period of 44-48 hours, RNA was extracted from the cells and reverse-transcribed to cDNA. Two different amounts of siRNA (50 pmol and 100 pmol) and three different volumes of Lipofectamine 2000 were tested.

RT-qPCR of cDNA from transfected cells was used to evaluate knockdown of *Ensa* mRNA, using primers specific to *Ensa* and a reference gene, *Gapdh*. Preliminary

experiments were conducted to investigate the efficiency of the primers. The efficiency of each primer set was between 90-110% and the R^2 values were > 0.99 (Figure 6). Although 50 pmoles of siRNA did not achieve significant knockdown at the ratios tested (data not shown), 100 pmoles of siRNA achieved ~50% knockdown of *Ensa* mRNA levels as compared with control cells when combined with 3 μL of Lipofectamine 2000 (Figure 7A).

Similar transfection experiments were conducted to examine knockdown at the protein level. Following a culture period of 44-48 hours, ENSA protein levels decreased by ~ 49% in cells treated with the *Ensa*-targeting siRNA as compared with control cells, which is consistent with the knockdown at the transcript level (Figure 7B; immunoblot performed by Lauren Matthews). Since Lipofectamine 2000 effectively delivered the *Ensa* siRNA, it served as a positive control in all transfection experiments.

Use of PEI or PEGylated PEI to introduce Ensa-targeting siRNA

Based on the gel mobility shift experiments with plasmid DNA (Figure 4), it appeared that PEI and PEG-PEI were capable of forming complexes with the plasmid at N/P ratios ≥10 and ≥ 5 respectively. To investigate the ability of PEI and PEG-PEI to deliver the *Ensa* siRNA, triplicates of NIH 3T3 cells were transfected with a complex consisting of PEI or PEG-PEI and the *Ensa* siRNA at an N/P ratio of 10, or with PEI or PEG-PEI alone (control cells). For comparison to a positive control, triplicates of cells were also transfected with a complex of Lipofectamine 2000 and the *Ensa* siRNA or with Lipofectamine 2000 alone (control cells). Following a culture period of 44-48 hours there was no significant change in *Ensa* mRNA levels in cells treated with the complex of

Ensa siRNA and PEI or PEG-PEI compared with control cells (Figure 8A). However, *Ensa* mRNA levels in cells treated with the complex of *Ensa* siRNA and Lipofectamine decreased by ~ 25% compared with control cells.

One explanation for the inability of PEI or PEG-PEI to achieve knockdown at an N/P ratio of 10, could be that the polymers failed to form complexes with the *Ensa* siRNA. To investigate complex formation further, we conducted the gel mobility shift assays with an RNA duplex that mimics the *Ensa* siRNA. While gel shift mobility assays indicated that both PEI and PEG-PEI could complex with plasmid DNA, the experiments performed with the RNA duplex suggested that PEG-PEI failed to form a complex with the duplex. With these results raising questions about the ability of PEG-PEI to form complexes with *Ensa* siRNA, we did not pursue further transfection experiments with the PEG-PEI, and instead focused on the delivery potential of unmodified PEI.

Because the gel mobility shift assays with the RNA duplex showed that PEI forms a complex with the duplex at N/P ratios ≥ 20, we wanted to assess whether PEI could deliver the *Ensa* siRNA into NIH 3T3 cells at an N/P ratio of 20. Thus, transfection experiments were conducted using an N/P ratio of 20 instead of 10. Following a culture period of 44-48 hours, *Ensa* mRNA levels in cells treated with *Ensa* siRNA and PEI only decreased by ~15% compared with control cells (Figure 8B), but the difference in *Ensa* mRNA levels was not statistically significant between cells treated with PEI + *Ensa*-targeting siRNA and cells treated with PEI only. On the other hand, in the same experiment, *Ensa* mRNA levels in cells treated with *Ensa* siRNA and Lipofectamine 2000 decreased by ~ 50%.

Use of HappyFect to Introduce the Ensa-targeting siRNA

Since PEI did not achieve significant knockdown under the conditions tested in these experiments, we investigated the ability of a commercially available polycation-based nanoparticle, HappyFect, to deliver the *Ensa* siRNA and knock down *Ensa* expression in NIH 3T3 cells. HappyFect was developed by scientists at Tecrea Ltd to overcome major limitations of existing transfection procedures, particularly poor efficiency and unacceptable levels of toxicity (information provided by the manufacturer, Tecrea Ltd). Combining HappyFect with RNA or DNA results in the rapid assembly of nanoparticles and the subsequent entry of the RNA or DNA into the cytoplasm of mammalian cells. HappyFect has been used in a wide variety of cell types (Tecrea Ltd). It can be used with even the most sensitive primary cells and the chemistry is compatible with *in vivo* applications (Tecrea Ltd).

Triplicates of NIH 3T3 cells were transfected with a complex consisting of HappyFect and the *Ensa* siRNA or with HappyFect alone. Two different amounts of HappyFect were tested. As a positive control, cells were also transfected with a complex consisting of Lipofectamine 2000 and the *Ensa* siRNA or with Lipofectamine 2000 alone. Following a culture period of 44-48 hours, *Ensa* mRNA levels in cells treated with *Ensa* siRNA and 10 µL of HappyFect in 2.5 mL of DMEM (0.4% HappyFect) decreased by ~9% compared with control cells; *Ensa* mRNA levels in cells treated with the *Ensa* siRNA and Lipofectamine 2000 decreased by ~26%, but neither result was statistically significant as error bars/confidence intervals overlap (Figure 9A). *Ensa* mRNA levels in cells treated with *Ensa* siRNA and 20 µL of HappyFect in 2.5 mL of DMEM (0.8% HappyFect) significantly decreased by ~36% compared with control cells, while *Ensa*

mRNA levels in cells treated with *Ensa* siRNA and Lipofectamine 2000 decreased by about 25%; knockdown with Lipofectamine 2000 in this experiment was not statistically significant (Figure 9B).

Similar transfection experiments were conducted to examine knockdown at the protein level, using 10 µL of HappyFect. Following a culture period of 44-48 hours, ENSA protein levels decreased by ~11% in treated cells compared with control cells (Figure 9C). I did not assess protein level knockdown using 20 µL of HappyFect because the cells had shown signs of toxicity when the transfection experiment was repeated.

4. Uptake of the FITC-Duplex using PEI as a Delivery Vehicle

The gel mobility shift assays indicated that PEI formed complexes with both plasmid DNA and a duplex that mimics the *Ensa* siRNA. However, when PEI was used to deliver the Ensa siRNA, at the N/P ratio (20) at which it formed a complex with the FITC-duplex, no significant knockdown was achieved. Althought it is possible that the PEI can form a complex with the FITC-duplex that mimicked the siRNA but not the actual *Ensa* siRNA, another explanation is that the polyplex was not actually endocytosed into the NIH 3T3 cells. To determine if the PEI was able to mediate cellular entry of nucleic acids at an N/P ratio of 20, I examined uptake of the polyplex formed with the FITC labeled duplex and PEI. As a positive control, uptake of a complex formed between Lipofectamine 2000 and the FITC-duplex was also examined. Duplex fluorescence could be seen 24 hours following the transfection in both NIH 3T3 cells transfected with PEI and Lipofectamine 2000 (Figure 10). However, some of the cells transfected with PEI and the FITC-oligo appear to show signs of cytoxicity.

5. Treatment of oocytes with HappyFect to introduce *Ensa*-targeting siRNA

Since HappyFect was the only polycation to achieve some knockdown (~36% at the transcript level), I wanted to test the ability of HappyFect to deliver the *Ensa* siRNA into mouse oocytes. Preliminary experiments were performed in which oocytes were transferred to a 35 μL drop of a 0.2 or 0.4% HappyFect solution. After a 4 hour treatment with the HappyFect, 24% (6/25) of the oocytes treated with the 0.2% HappyFect solution had died or appeared unhealthy, while 44% (11/25) of the oocytes treated with the 0.4% HappyFect solution had died or appeared unhealthy. Thus, to introduce the *Ensa* siRNA into oocytes, the 0.2% HappyFect solution was chosen for the transfection protocol.

For the transfection, 60 oocytes were treated with a complex of HappyFect and the *Ensa* siRNA, and 60 oocytes were treated with a complex of HappyFect and a negative control siRNA. As an additional control, 20 oocytes were cultured in parallel in plain WB-medium; these cells were assessed periodically, to compare the visual appearance of these oocytes in the absence of HappyFect to oocytes in the presence of HappyFect. Following a culture period of 44-48 hours, 31% of the oocytes (19/60) treated with the negative control siRNA and 18% of the *Ensa* siRNA-treated oocytes (11/60) had died or appeared unhealthy; all of the untreated oocytes remained healthy/alive. An immunoblot analysis of the protein levels in 20 untreated oocytes, 20 oocytes treated with HappyFect and *Ensa* siRNA, and 20 oocytes treated with *HappyFect* and negative control siRNA, showed that no knockdown of ENSA was achieved as the intensity of both the ENSA and GAPDH (loading control) bands were approximately equivalent in all three groups (Figure 12).

Discussion

1. Requirements of Polycation-mediated Nucleic Acid Delivery

The delivery of nucleic acids using polycations is a multi-faceted process that is dependent on the condensation of DNA, cellular uptake, release of the nucleic acid from the polyplex, and many other factors. Recent studies have shown that the efficiency of polycation-mediated delivery varies with the N/P ratio; particularly, transfection efficiency has been shown to increase with the use of excess polycations (Boeckle et al., 2004; Dai, Gjetting, Mattebjerg, Wu, & Andresen, 2011). However, since increasing N/P ratios incurs toxic effects on cells, cytotoxicity is another important consideration. Successful siRNA-mediated knockdown requires the optimization of all the features that influence polycation-mediated delivery as well as the essential features intrinsic to RNAi. According to my results, although the commercially available polycation-based nanoparticle HappyFect was able to knock down *Ensa* in NIH 3T3 cells at the transcript level (Figure 9B), neither PEI nor PEGylated PEI was able to knock down *Ensa* transcripts. However, HappyFect was unable to knock down ENSA protein levels in mouse oocytes, under the conditions tested in this thesis (Figure 11). These results may be explained by the inability of the polycations to meet any one of the essential requirements of effective polycation-mediated delivery.

1.1. Nucleic Acid Condensation

The condensation of DNA or RNA into small particles by polycations is necessary for the efficient delivery of nucleic acids into cells. Because nucleic acid condensation is dependent on electrostatic interactions between the polycation and DNA

or RNA, complex formation is a function of the N/P ratio. However, along with the N/P ratio, complex formation is also dependent on a number of other factors as well, including the charge density of the polymer and the ionic strength of the solvent in which polyplexes are formed.

The binding of nucleic acids to PEI is thought to be mainly driven by entropic forces arising from the release of counter ions (Neu et al., 2005). Polycations with a high charge density, such as PEI, can release more counter ions upon binding with DNA and therefore form more stable complexes (Bronich, Kabanov, & Marky, 2001). Moreover, a lower charge density has been shown to impair the condensation capability of polycations (Wolfert & Seymour, 1996). PEGylation of PEI provides steric shielding of the PEI moiety, to improve complex solubility (Kichler, Chillon, Leborgne, Danos, & Frisch, 2002) and aggregation (Tang et al., 2003), and to reduce the interaction between PEI and proteins or other negatively charged components in cells (Tang et al., 2003). As a result, PEGylated PEI tends to be less toxic than unmodified polymers *in vitro* and *in vivo* (Petersen et al., 2002). However, since a high positive charge density is necessary for the condensation of DNA, the addition of PEG can alter the complex-forming behavior of PEI and render DNA condensation more difficult, due to the steric layer that shields the charged PEI. In particular, studies have shown that while PEG conjugates with molecular weights ranging from 350-1900 Da (Sung et al., 2003) did not have a significant effect on the complex-forming behavior as a function of the N/P ratio, the complex-forming ability of PEIs grafted to a varied number of higher MW PEGs (5 kD) was hindered slightly (Petersen et al., 2002). Gel mobility shift assays with the PEGylated PEI used in this thesis showed that while PEI formed a complex with an RNA duplex that mimics siRNA,

PEGylated PEI failed to form a complex with the duplex (Figure 5). One possible explanation for these results is that the steric stabilization of PEI by the PEG conjugates reduced the charge density of PEI and therefore hindered polyplex formation. Moreover, the duplex that was used to mimic the siRNA contained an oligo-2'-O-methylribonucleotide annealed to a complementary RNA; the methyl group may have contributed to the reduction of the charge density of the polyplex, further impeding complex formation. Although this raises the question of whether it is possible that the PEG-PEI was able to form a complex with the *Ensa* siRNA but not the RNA duplex, the inability of PEG-PEI to transfect NIH 3T3 cells is convincing evidence that the PEGylated PEI does not form a complex with either the RNA duplex or the *Ensa* siRNA.

Another important factor of DNA condensation is the medium in which complexes are prepared. The size of PEI/DNA complexes formed in saline solutions is thought to be dependent on the ionic strength of the solution, and the tendency of complex sizes to increase with increasing saline concentration is thought to reflect a decreased binding affinity between polycations and nucleic acids (Kabanov & Kabanov, 1998; Ogris et al., 1998). Because of the reduced binding affinity, higher salt concentrations can result in the dissociation of polyplexes; as a result, it is possible that PEGylated PEI did not form a complex with either the *Ensa* siRNA or the RNA duplex due to the high ionic strength of the solution in which the complexes were prepared. One study reported a drastic decrease of complex size when comparing complexes prepared in physiological salt solution with those in 5% glucose (Goula et al., 1998). Future experiments could involve changing the conditions of the medium in which the complexes of PEGylated PEI and the *Ensa* siRNA are formed.

Additionally, as reported by Zintchenko et al., since siRNAs are very small molecules, the limited electrostatic interaction with polycations to achieve sufficient complex stability is a general problem for cationic synthetic carriers (2008). One study has shown that the stability of branched PEI polyplexes against salt dissociation is nearly two times higher for plasmid DNA as compared to siRNA polyplexes (Zintchenko, Philipp, Dehshahri, & Wagner, 2008). Since PEGylated PEI formed a complex with the plasmid DNA but not the RNA duplex, it is possible that the polyplexes formed between PEG-PEI and the pGEM-3Z plasmid were stable enough to prevent dissociation caused by high salt concentrations, but the polyplexes between PEG-PEI and the RNA duplex were unstable and dissociated due to the ionic strength of the medium in which they were prepared.

1.2 Cellular Uptake

The transfection efficiency of polyplexes can vary largely depending on the cell type (Gebhart & Kabanov, 2001), possibly due to differences in endocytosis and intracellular transport of polyplexes. Fluorescence microscopy experiments investigating the delivery of a FITC-labeled duplex into NIH 3T3 cells using PEI as a delivery vehicle suggested that the PEI was capable of mediating cellular entry of a duplex (Figure 10). Knockdown of *Ensa* mRNA levels in cells transfected with HappyFect and the Ensa siRNA (Figure 9B) indicated that HappyFect was also able to mediate cellular entry of siRNA.

The ability of HappyFect to introduce nucleic acids into oocytes could rely on a number of factors. The positive surface charge of polycation/DNA complexes serves to

bind to cells via electrostatic interactions with negatively charged proteoglycans of the cell membrane. Recent studies have indicated that these interactions with proteoglycans may also be essential for the subsequent process of internalization of the polyplexes (Uyechi, Gagne, Thurston, & Szoka, 2001). As a result, a greater density of negatively charged proteoglycans on the surface of a particular cell type is believed to enhance polycation-mediated delivery (Kircheis, Wightman, & Wagner, 2001a). Some cell types, such as melanoma cells, have been shown to achieve better uptake of positively charged PEI/DNA complexes because of a large presence of negatively charged cell-surface proteoglycans (Kircheis et al., 2001a). Additionally, to enhance cellular uptake and increase transfection efficiency, efforts have been made to combine the non-specific electrostatic polyplex-cell surface interaction with the specific mechanism of receptor-mediated cellular uptake by incorporating cell-binding ligands into transfection complexes (Kircheis, Wightman, & Wagner, 2001b). The ability of HappyFect to mediate cellular entry of siRNAs into oocytes may depend on the proteoglycans on the cell membrane, and incorporating an oocyte cell-binding ligand could improve cellular uptake of polyplexes.

Additionally, the zona pellucida has been shown to act as a barrier in transfections of oocytes using lipid-based delivery vehicles (Carballada, Relloso, & Esponda, 2002); it is possible that the ZP may be a potential barrier for HappyFect in delivering the *Ensa* siRNA into oocytes and that removal of the ZP may be necessary for polycation-mediated delivery in oocytes.

1.3. Release of Nucleic Acid from the Polyplex

For RNAi mediated knockdown to occur, once polyplexes are released from endosomes, the siRNA must enter the cytosol and associate with its complementary mRNA to either target cleavage of the mRNA or translational arrest. To guarantee biological activity of the siRNA, polyplexes should be sufficiently stable during endocytosis to ensure intracellular uptake of the siRNA, but the siRNA must also be released from the polyplex into the cytosol for subsequent association with its target. PEGylation of PEI has been shown to destabilize PEI/siRNA polyplexes, leading to increased siRNA release in the cytoplasm and therefore more efficient knockdown (Petersen et al., 2002). Although the PEGylated PEI used in this thesis research failed to form complexes with the RNA duplex mimicking the *Ensa* siRNA, the PEI readily formed complexes with both the plasmid DNA and the RNA duplex. One possible explanation for the inability of PEI to knockdown *Ensa* mRNA in NIH 3T3 cells could be that the complexes of PEI and the *Ensa* siRNA were too stable, preventing the release of the *Ensa* siRNA from the polyplexes inside the cytosol and its association with *Ensa* mRNA. Other polycations may be feasible alternatives. Novel arginine grafted bioreducible polymers, for example, have been engineered to electrostatically interact with negatively charged siRNA, form stable complexes, and to release siRNA into the cytosol through intracellular cleavage of a bioreducible backbone (Kim, Jeong, Kim, Kim, & Bull, 2009).

1.4. Balance between cytotoxicity and transfection efficiency

Generally, the N/P ratio at which polyplexes are formed is not necessarily the optimal N/P ratio for transfection. For efficient transfection, PEI polyplexes are usually generated with an excess of PEI, where a considerable amount of PEI is free in solution (Clamme, Azoulay, & Mely, 2003; Finsinger, Remy, Erbacher, Koch, & Plank, 2000). Boeckle et al., purified polyplexes, consisting of PEI and plasmid DNA, through size exclusion chromatography to remove free polycations and transfected cells with either purified polyplexes or non-purified polyplexes (2004). Not only was the transfection efficiency of non-purified particles greater than that of purified particles, but also the addition of free PEI to cells treated with purified particles resulted in significantly enhanced transfection efficiency (Boeckle et al., 2004). When NIH 3T3 cells were transfected with PEI and the *Ensa* siRNA at an N/P ratio of 20, no significant knockdown was achieved; it is possible that increasing the N/P ratio would achieve greater knockdown due to enhanced transfection efficiency facilitated by free PEI. However, the fluorescence microscopy experiments showed that when the cells were transfected with a complex of PEI and a FITC-labeled duplex at an N/P ratio of 20, the cells appeared unhealthy and showed signs of toxicity. Since an increased N/P ratio in polycation-mediated transfection directly correlates with cellular toxicity (Godbey, Wu, & Mikos, 2001; Morimoto et al., 2003), a desirable balance between increased transfection efficiency and reduced cytotoxicity must be maintained.

This need for a balance between transfection efficiency and cytotoxicity also holds true for the delivery of the *Ensa* siRNA using HappyFect, in both NIH 3T3 cells and oocytes. While using 10 uL of HappyFect did not achieve much knockdown in NIH

3T3 cells at the transcript or protein level, 20 uL of HappyFect did achieve significant knockdown at the transcript level (Figure 9). However, 20 uL of HappyFect appeared to have toxic effects on the cells when the experiment was repeated. Before treating oocytes with HappyFect and the *Ensa* siRNA, I examined the toxicity of two different concentrations of transfection mixtures, containing 0.2 or 0.4% HappyFect in WB medium). In NIH 3T3 cells, 0.4 or 0.8% HappyFect solutions in serum-free DMEM were used for the transfections, with the 0.8% HappyFect solution having toxic effects on the cells when the experiment was repeated. However, although a 0.4% HappyFect solution did not seem to have toxic effects on NIH 3T3 cells, both 0.2 and 0.4% HappyFect solutions appeared to have toxic effects on the oocytes. It is possible that increasing the amount of HappyFect used for the oocyte experiments could improve the transfection efficiency of the *Ensa* siRNA. However, future cytotoxicity studies, such as the use of an MTT assay, may provide more information about achieving a balance between transfection efficiency and cytotoxicity.

1.5. Efficiency of the *Ensa* siRNA

RNAi is dependent on the effective delivery of shRNA or siRNA into cells as well as the ability of shRNA or siRNA to effectively associate with its complementary target mRNA and facilitate RNA degradation or translational arrest. While microinjection of the *Ensa* siRNA in mouse oocytes achieved a range of 39-91% knockdown in five experiments (Matthews & Evans, 2014), using Lipofectamine 2000 to deliver the *Ensa* siRNA cocktail into NIH 3T3 cells was only able to knock down *Ensa* mRNA levels by about 25-50%. While 100 pmoles, which was the amount of siRNA used for all

experiments, may not be sufficient for knockdown greater than 50%, it is also possible that the siRNA is not as efficient in the NIH 3T3 cells as it is in oocytes. For a better evaluation of the delivery potential of polycations, it might be beneficial to use a siRNA that is as efficient in the positive control cells that we are using (NIH 3T3) as in oocytes.

2. Applications of an Alternative Nucleic Acid Delivery Vehicle in Oocytes

Although there are several considerations for polycation-mediated delivery of siRNA or shRNA into oocytes, the impact of an alternative method to microinjection would be tremendous for the field of oocyte biology. Particularly, developing a delivery vehicle to knock down targets that are important in oocyte maturation, such as ENSA, could have applications in non-hormonal contraception.

ENSA, alpha-endosulfine, plays a regulatory role in meiosis. Mammalian oocytes progress through meiosis in a staggered fashion. Upon ovulation, oocytes undergo meiotic maturation, which is the progression from prophase I to metaphase of meiosis II. The exit from meiotic arrest, or re-entry into meiosis II is regulated by a collection of signaling proteins. Cyclin-dependent kinase 1 (CDK1) phosphorylates various substrates that stimulate events associated with re-entry into M-phase (Gharbi-Ayachi et al., 2010). Along with the phosphorylation of these substrates, the inhibition of protein phosphatase 2A (PP2A) is essential for regulation of meiotic re-entry (Gharbi-Ayachi et al., 2010). ENSA is one protein that dephosphorylates PP2A and is therefore a key regulator of entry into meiosis (Gharbi-Ayachi et al., 2010). Moreover, previous studies have identified physiological roles for ENSA in mouse and *Drosophila* oocytes. *Endos* mutant *Drosophila* oocytes and ENSA-deficient mouse oocytes both exhibit defects in

progression out of prophase I arrest (Matthews & Evans, 2014; Von Stetina et al., 2008). The essential role that ENSA plays in the regulation of meiosis in oocytes makes it a potential target for future non-hormonal contraceptive applications. Thus, developing a nanoparticle system that could circumvent microinjection and deliver gene disrupting agents into oocytes would not only transform mammalian oocyte research by elucidating unknown biological processes, but could also have long term implications for human contraception through the disruption of genes necessary for oocyte growth and maturation.

3. Future Directions

The cytotoxicity of polycations is a major barrier in polycation-mediated transfections. Both HappyFect and PEI incurred cytotoxic effects on NIH 3T3 cells and oocytes. Although PEGylation is believed to reduce the cytotoxicity of PEI by providing a surface charge shield, it is possible that it may hinder the complex-forming behavior of PEI. Other measures have been developed to reduce the cytotoxicity of PEI. Acylation of the amino groups on the backbone of PEI has been shown to improve its transfection efficiency and reduce cytotoxicity (Aravindan, Bicknell, Brooks, Khutoryanskiy, & Williams, 2009; Thomas & Klibanov, 2002). The PEG that I used in this thesis was derivatized with a maleimide amine; it is possible that acylating amino groups of either the PEI or the amino groups of the PEG-maleimide-amine may reduce cytotoxicity.

Future experiments could also involve the investigation of other polycations to deliver nucleic acids into oocytes since PEI, PEGylated PEI, and HappyFect were not as effective in delivering the siRNA into NIH 3T3 cells as Lipofectamine. Along with PEI,

polyamidoamine (PAMAM) dendrimers are among the most extensively studied and widely used polycations. They are made of repetitively branched subunits of amide and amine functional groups. The relative low cost of synthesis of PAMAM dendrimers, along with their biocompatibility and structural control have made them candidates for applications in drug delivery (Kukowska-Latallo et al., 1996; Tomalia, Reyna, & Svenson, 2007). Unlike PEI, which is nondegradable, PAMAM dendrimers are biodegradable polymers with a backbone of polymer chains consisting of peptide bonds; as a result, they exhibit comparatively less cytotoxicity than PEI (Choi, Kang, Kim, Lim, & Chung, 2010).

Additionally, since cellular uptake is another potential barrier for introducing nucleic acids into oocytes, the incorporation of an oocyte-specific cell ligand into the transfection complex could enhance transfection. Particularly, combining a DNA or RNA aptamer that specifically binds to the zona pellucida of oocytes with a polycation delivery vehicle would allow us to introduce siRNA or shRNA into ZP-intact oocytes and could have applications in drug delivery or gene therapy in mammalian oocytes.

Moles of Phosphate (Constant)	6.1×10^{-11}	
N/P Ratio	Moles of Nitrogen	Moles of PEI/PEG-PEI
1	6.1×10^{-11}	1.04×10^{-13}
5	3.05×10^{-10}	5.2×10^{-13}
10	6.1×10^{-10}	1.04×10^{-12}
15	9.15×10^{-10}	1.57×10^{-12}
20	1.22×10^{-9}	2.09×10^{-12}

Table 1. Molar amounts of PEI or PEGylated PEI used for gel mobility shift experiments with pGEM-3Z plasmid. 20 ng of plasmid DNA (61 pmoles of phosphate) were combined with different molar amounts of polycation according to the N/P ratio.

Moles of Phosphate (Constant)	1.8×10^{-10}	
N/P Ratio	Moles of Nitrogen	Moles of PEI/PEG-PEI
5	9×10^{-10}	1.54×10^{-12}
10	1.8×10^{-9}	3.09×10^{-12}
15	2.7×10^{-9}	4.63×10^{-12}
20	$3.6x\ 10^{-9}$	6.17×10^{-12}
25	4.5×10^{-9}	7.72×10^{-12}
30	5.4×10^{-9}	9.26×10^{-12}
35	6.3×10^{-9}	1.08×10^{-12}
40	7.2×10^{-9}	1.23×10^{-12}

Table 2. Molar amounts of PEI or PEGylated PEI used for gel mobility shift experiments with RNA duplex containing a FITC labeled oligo-2'-O-methylribonucleotide annealed to a complementary RNA. 5 pmoles of the duplex (180 pmoles of phosphate) were combined with different molar amounts of polycation according to the N/P ratio.

***Ensa*-siRNA Pool Target Sequences**
GCGAGGAGAAGCAGGAUAC
GCUCGUCACCAGCAAGCUU
GAAGAGACUCCAGAAAGGG
GCUAAAGGCCAAAUAUCCA

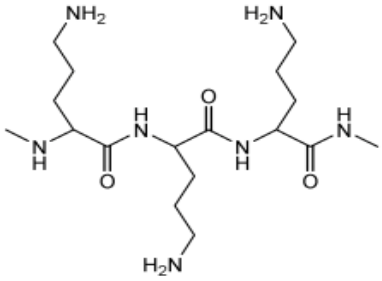
Table 3. *Ensa* mRNA sequences targeted by the *Ensa* ON-TARGETplus SMARTpool siRNA (Thermo Scientific) used for all knockdown experiments.

Plate	Media (vol/well)	Transfection Mixture Volume (µL)	siRNA (pmol)	HappyFect
24-well	500 µL	25	10	2
12-well	1 mL	50	20	4
6-well	2.5 mL	125	50	10
60-mm	5 mL	250	100	20

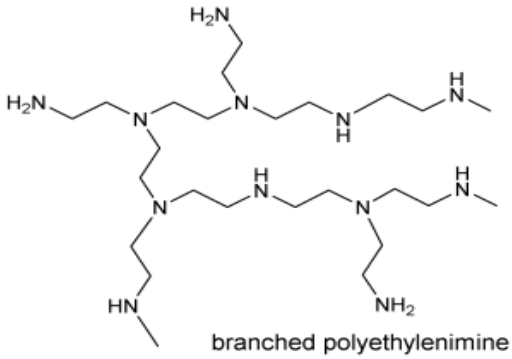
Table 4. Suggested volumes and amounts of reagents for transfections using HappyFect (Tecrea Ltd). According to manufacturer's instructions, amounts of siRNA and HappyFect can be varied +/- 50%. For transfections in NIH 3T3 cells the 6-well plate format was used, with 10 or 20 µL of HappyFect. Suggested amounts for the 24-well plate format were used for treatment of oocytes with HappyFect, with 1 µL of HappyFect instead of 2 µL.

Primer	***Gapdh***	***Ensa***
Forward Sequence (5'→3')	AGGTCGGTGTGA ACGGATTTG	GAAAACCCTGCGG AGGAGACCG
Reverse Sequence (5'→3')	TGTAGACCATGT AGTTGAGGTCA	CCCGCAAGCTTGC TGGTGAC

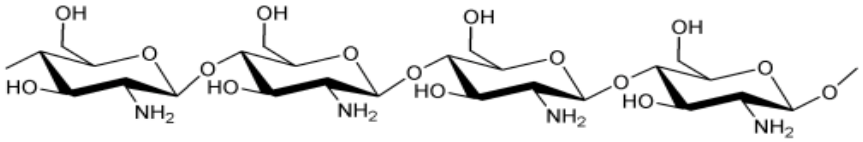
Table 5. Primer sequences used for RT-qPCR experiments evaluating levels of *Ensa* (gene of interest) and *Gapdh* (reference gene) in cells transfected with an *Ensa*-targeting siRNA.



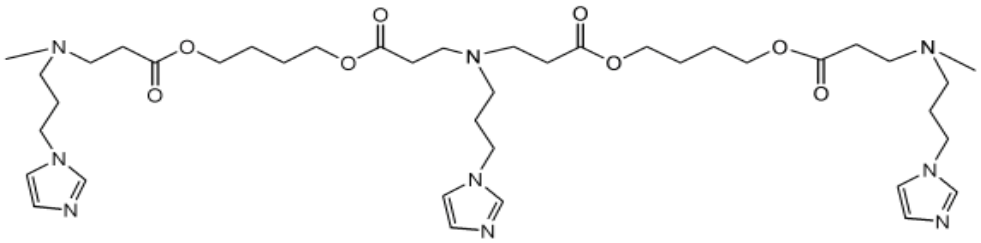
polylysine



branched polyethylenimine

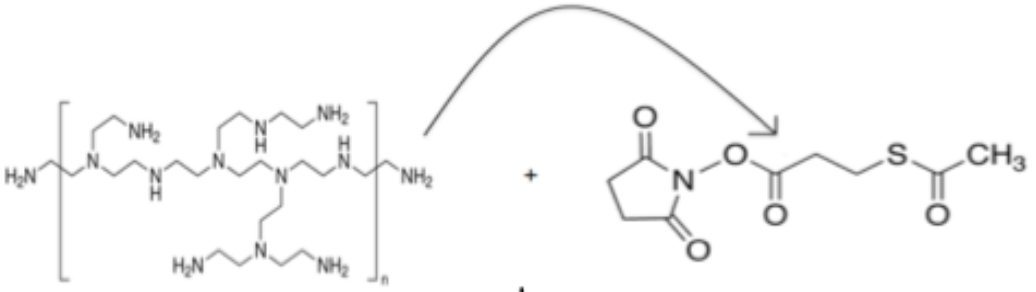


chitosan



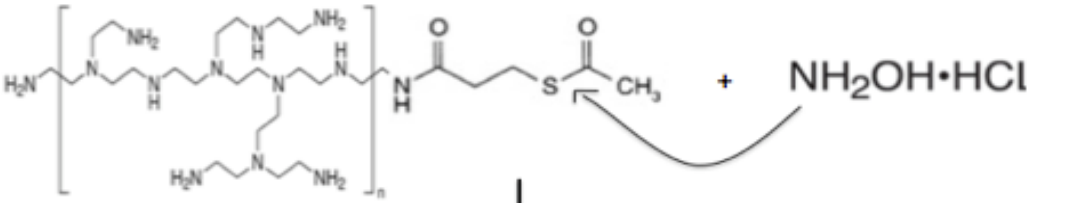
a poly(β-amino ester)

Figure 1. Chemical structures of a few representative polycations used for nucleic acid delivery (Thomas & Klibanov, 2003). Branched polyethyleneimine, explored in this thesis, is one of the most effective delivery agents due to its ability to act as a proton sponge and facilitate endosomal escape of a polyplex into the cytosol.



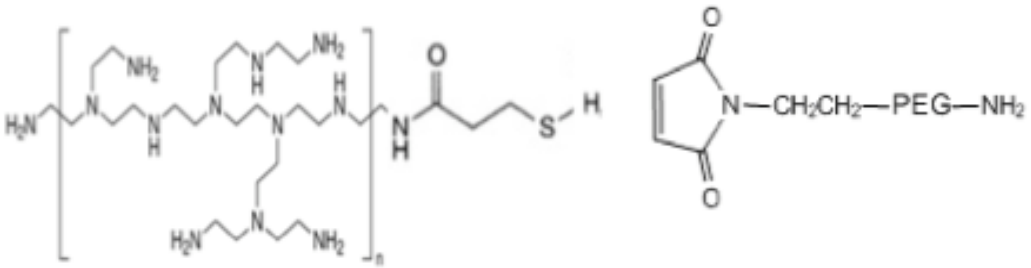
Branched Polyethyleneimine (PEI)
MW = 25 kD

N-Succinimidyl-S-acetylthiopropionate
(SATP)



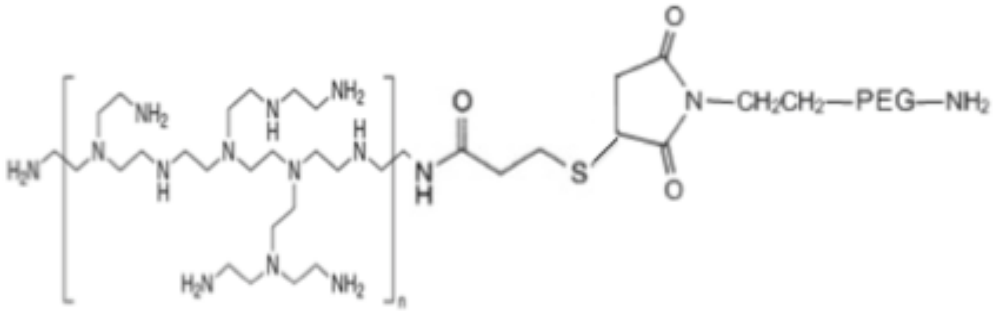
Acetylated PEI with Protected
Sulfhydryl Group

Hydroxylamine Hydrochloride



Deacetylated PEI with Thiol Group

Maleimide PEG Amine



Polyethylene gycol-Polyethyleneimine
Molar Ratio of PEG: PEI = 10:1

Figure 2. Schematic of the synthesis of PEGylated PEI. Conjugation of PEG to PEI involves 3 steps. Step 1 is the addition of an SATP linker to form an acetylated PEI molecule with a protected sulfhydryl group. Step 2 is the deacetylation of the PEI molecule using hydroxylamine hydrochloride in order to free the thiol group. Step 3 involves the selective reaction between the free thiol group attached to PEI with the maleimide group of the PEG derivative used in this project. The molar ratio of PEG to PEI was 10:1.

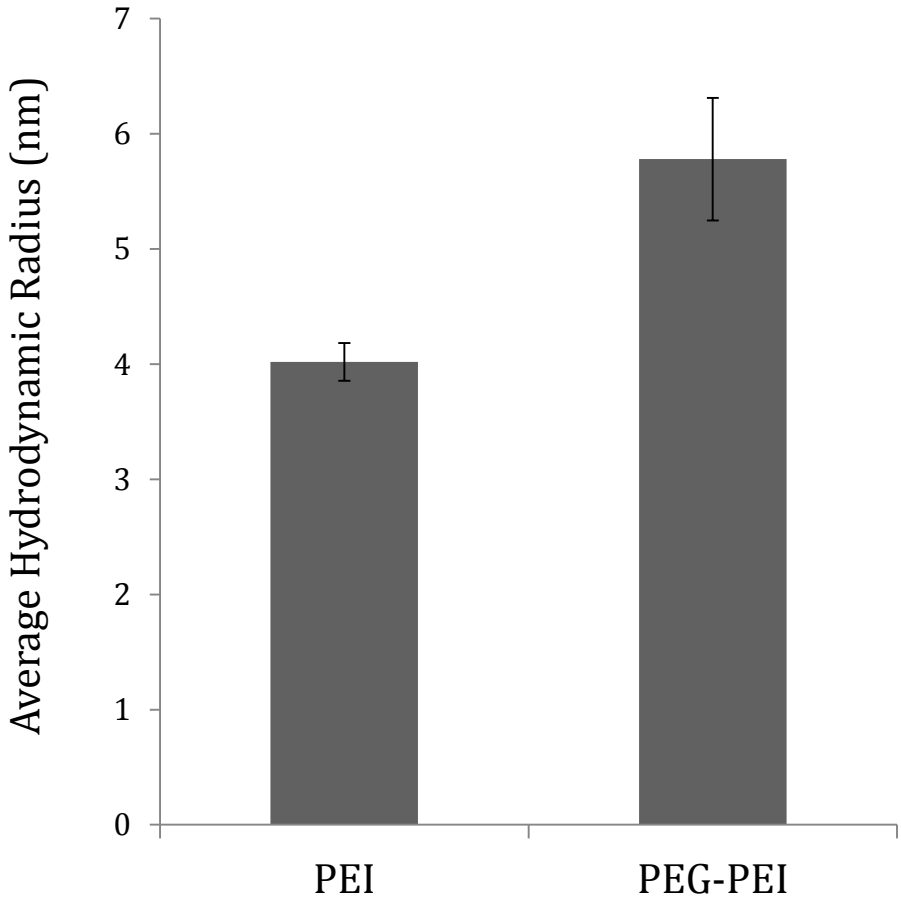
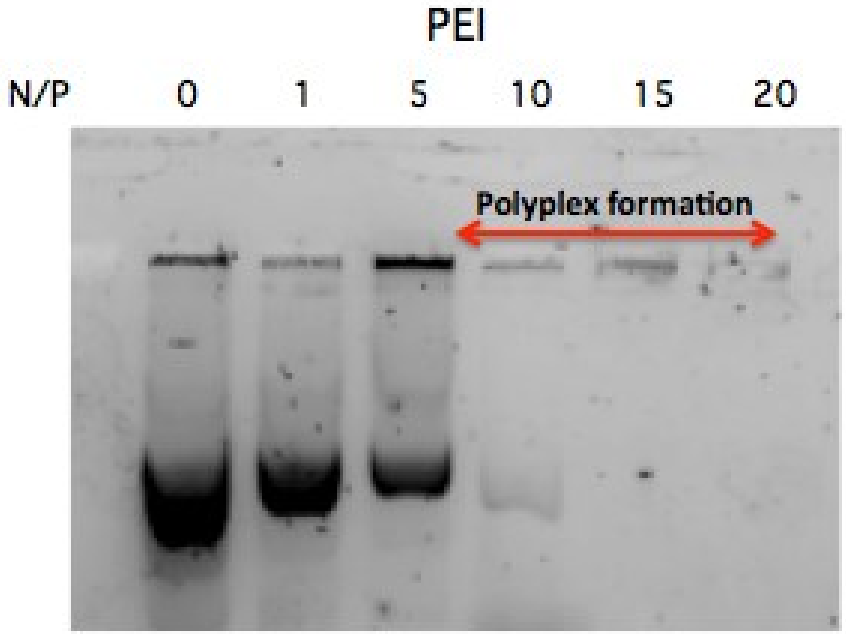

Average Hydrodynamic Radius (nm)
7
6
5
4
3
2
1
0
PEI
PEG-PEI

Figure 3. Graph of the size of particles of a solution of unmodified PEI or PEGylated PEI, as determined by dynamic light scattering. The y-axis shows the average hydrodynamic radius of 5 measurements (5 different aliquots of one solution). The standard error represents variation between each measurement.


PEI
N/P
0
1
5
10
15
20
Polyplex formation

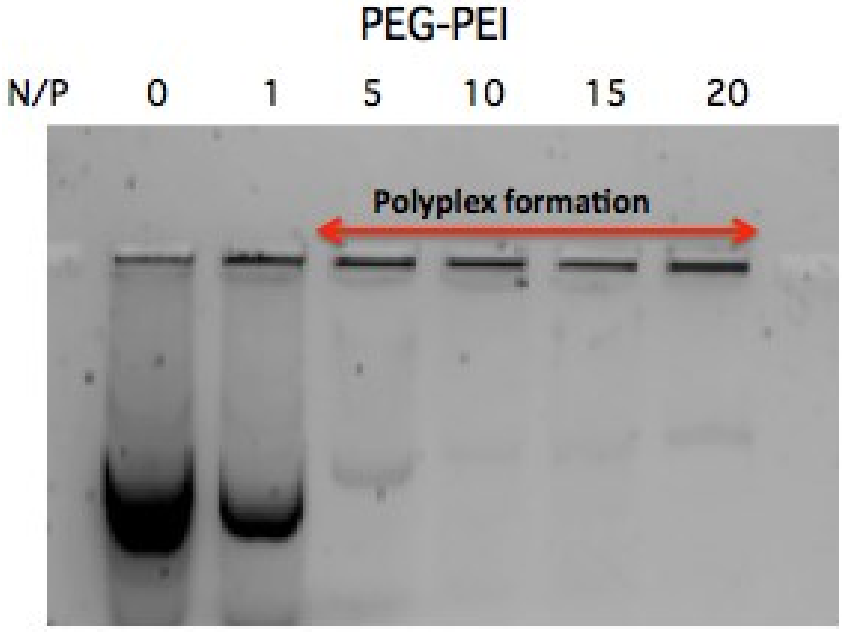
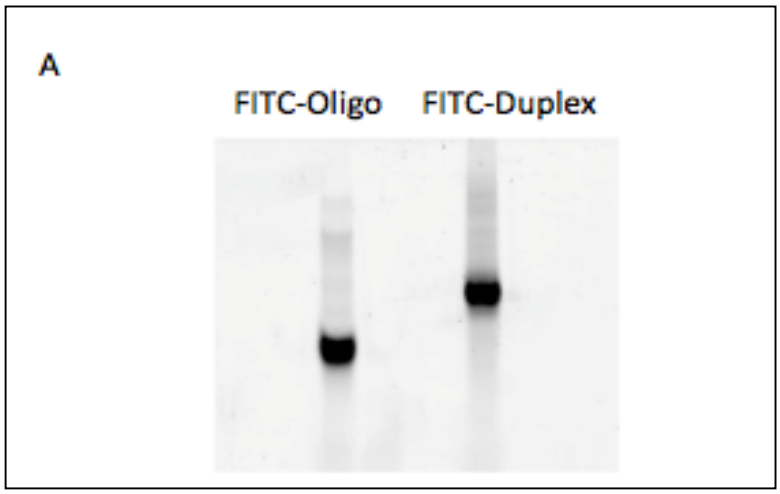

PEG-PEI
N/P
0
1
5
10
15
20
Polyplex formation

Figure 4. 0.8% agarose gels, containing SYBR green dye, loaded with solutions of pGEM-3Z and PEI or PEGylated PEI at varying N/P ratios. Each lane is loaded with a mixture of plasmid and polycation with a different N/P ratio (see Table 1 for specific molar amounts). An N/P ratio of 0 indicates that only the plasmid (without the polycation) was loaded. Disappearance of the SYBR green signal signifies complex formation due to exclusion of the SYBR green dye from the polyplex.


A
FITC-Oligo
FITC-Duplex

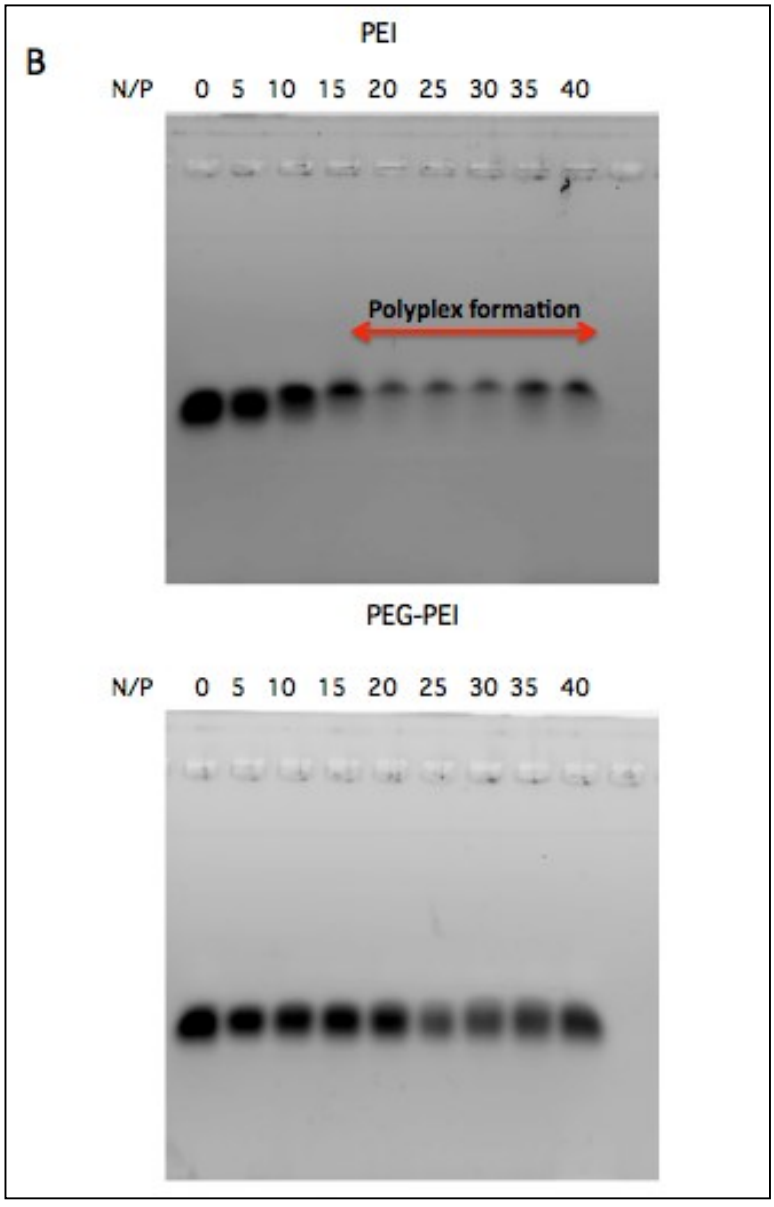
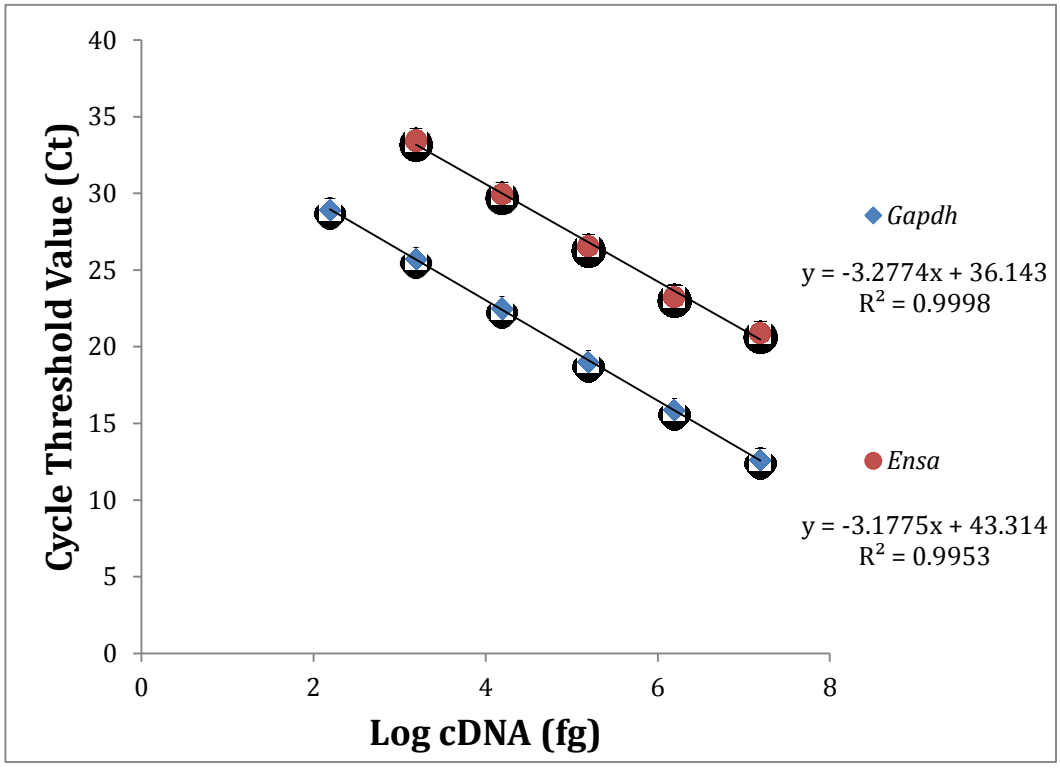

B
PEI
N/P 0 5 10 15 20 25 30 35 40
Polyplex formation
PEG-PEI
N/P 0 5 10 15 20 25 30 35 40

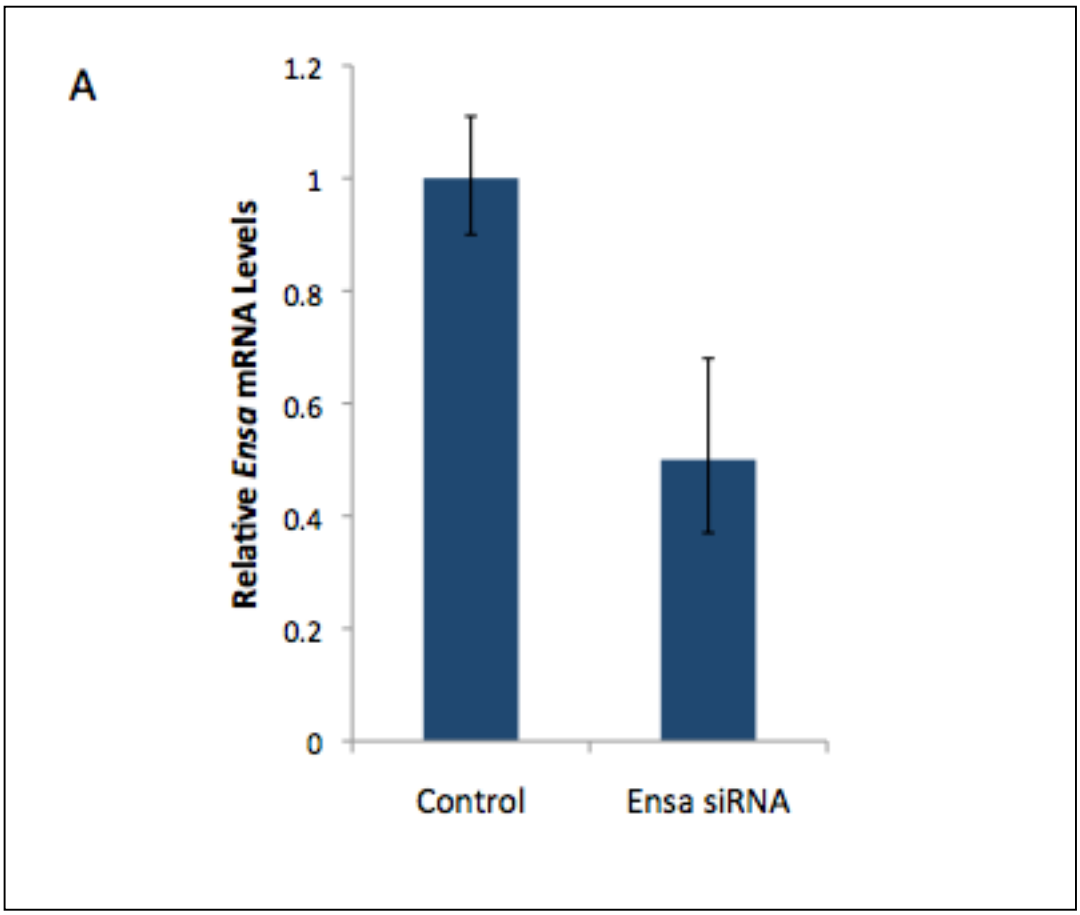
Figure 5. A) A 20% native polyacrylamide gel loaded with a FITC labeled oligo-2'-O-methylribonucleotide alone (FITC-Oligo) or the oligo annealed to a complementary RNA (FITC-Duplex). **B)** 1% agarose gels loaded with solutions of the FITC-duplex and PEI or PEGylated PEI at varying N/P ratios. Each lane is loaded with a mixture of the duplex and a polycation with a different N/P ratio (see table 2 for specific molar amounts). An N/P ratio of 0 indicates that only the duplex (without the polycation) was loaded. Quenching of the fluorescence of the FITC signal indicates polyplex formation.



Efficiency of *Gapdh* Primer Set = $(-1 + 10^{(-1/-3.2774)}) * 100$ = **101.9%**

Efficiency of *Ensa* Primer Set = $(-1 + 10^{(-1/-3.1775)}) * 100$ = **106.5 %**

Figure 6. Standard curve for *Gapdh* and *Ensa* primers generated by preparing serial dilutions of cDNA from NIH 3T3 cells. Cycle threshold is on the y-axis and the log of the template concentration (set as fg; estimated from RNA input) is on the x-axis. Slope of the curves are used to calculate the efficiency of each primer set.


A
1.2
1
0.8
0.6
0.4
0.2
0
Relative Ensa mRNA Levels
Control
Ensa siRNA

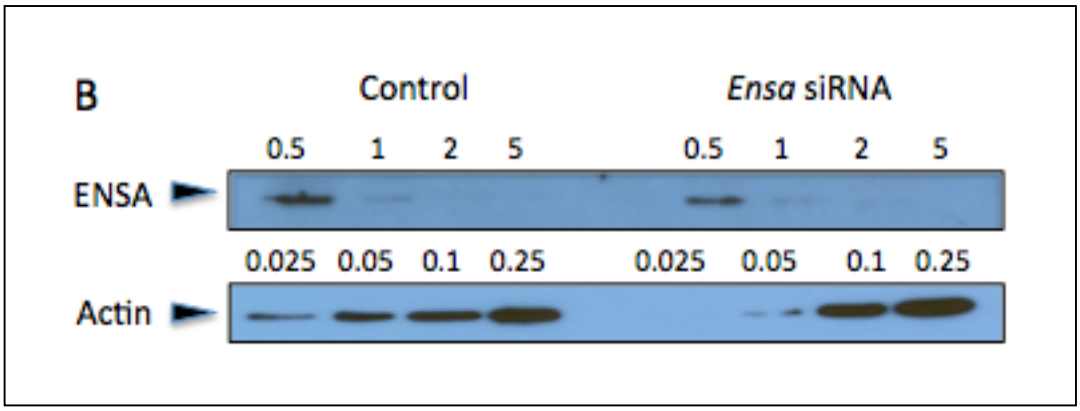
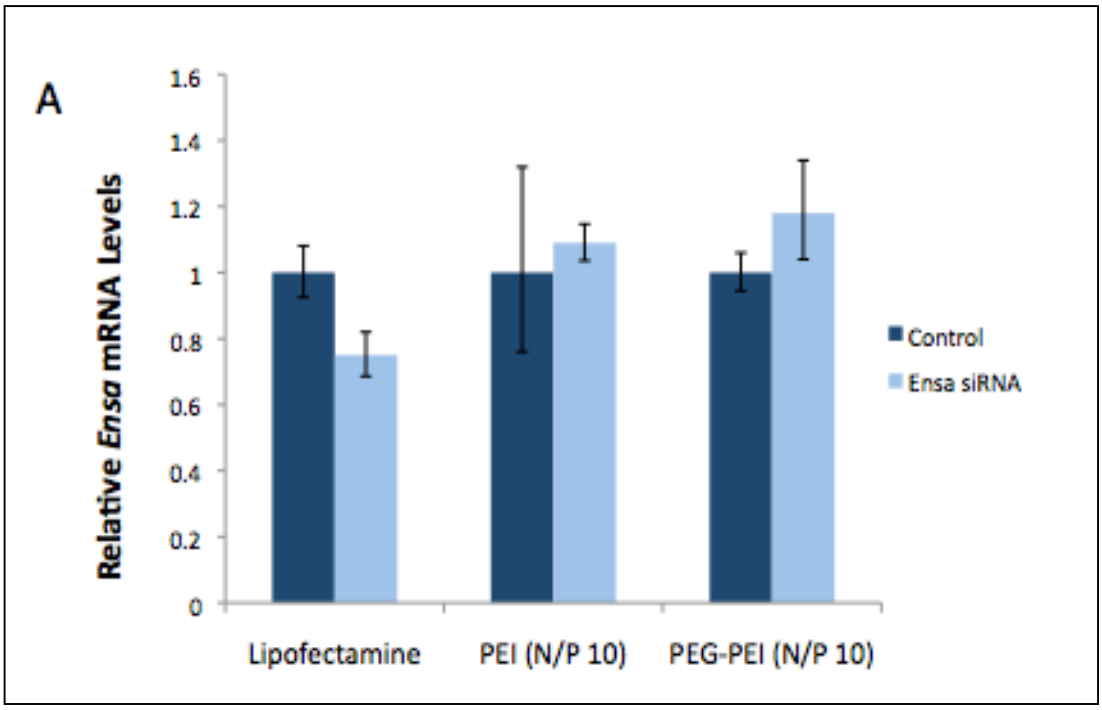

B
Control
Ensa siRNA
0.5
1
2
5
0.5
1
2
5
ENSA
0.025
0.05
0.1
0.25
0.025
0.05
0.1
0.25
Actin

Figure 7. A) Levels of *Ensa* transcript in NIH 3T3 cells, 44-48 hours following a transfection using Lipofectamine as a delivery vehicle. The graph shows relative *Ensa* mRNA levels of cells transfected with a complex of Lipofectamine and *Ensa* siRNA (*Ensa* siRNA on x-axis) or Lipofectamine alone (control on x-axis). Relative *Ensa* mRNA levels are normalized to a reference gene, *Gapdh*. Error bars represent a pooled standard error of the variation in Ct values between triplicates for each primer set, as well as the variation between each experimental group (control and *Ensa* siRNA). **B)** Levels of ENSA protein in NIH 3T3 cells, 44-48 hours following a transfection using Lipofectamine as a delivery vehicle. Western blots using cell lysate from control cells (Lipofectamine only) or *Ensa* siRNA treated cells (Lipofectamine + *Ensa* siRNA were prepared by probing for ENSA (top) and Actin (bottom) separately. Each blot shows protein bands for different amounts of cell lysate for each experimental group. The numbers above each blot represent the volume of cell lysate (μL) loaded in each well of the polyacrylamide gel. To stay within linear range, ENSA protein levels in 0.5 μL of cell lysate were normalized to levels of Actin in 0.1 μL of cell lysate for calculating knockdown.


A
Relative Ensa mRNA Levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Lipofectamine
PEI (N/P 10)
PEG-PEI (N/P 10)
Control
Ensa siRNA

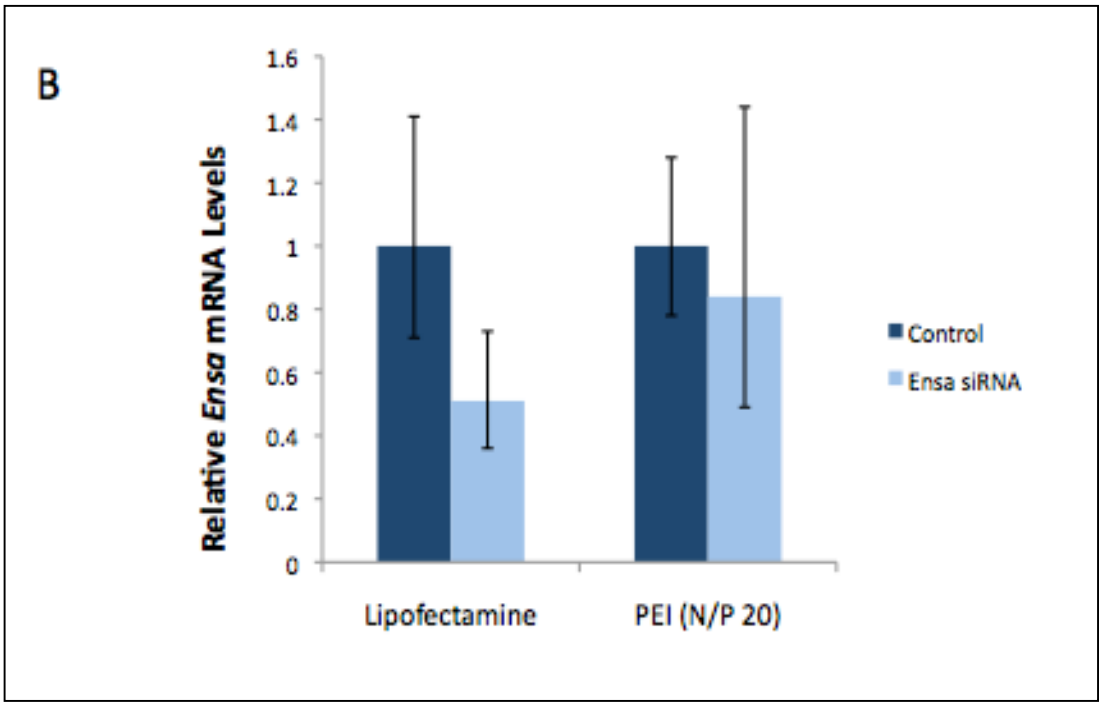
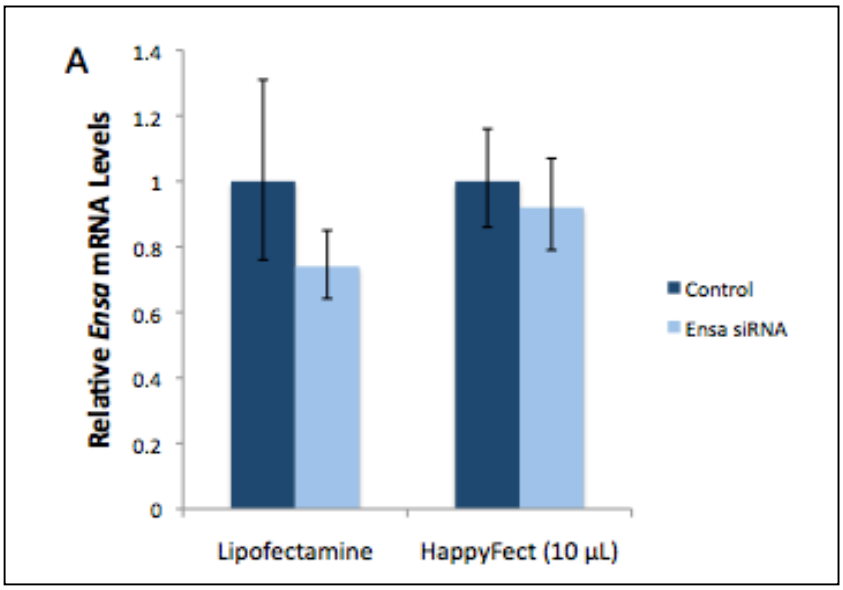
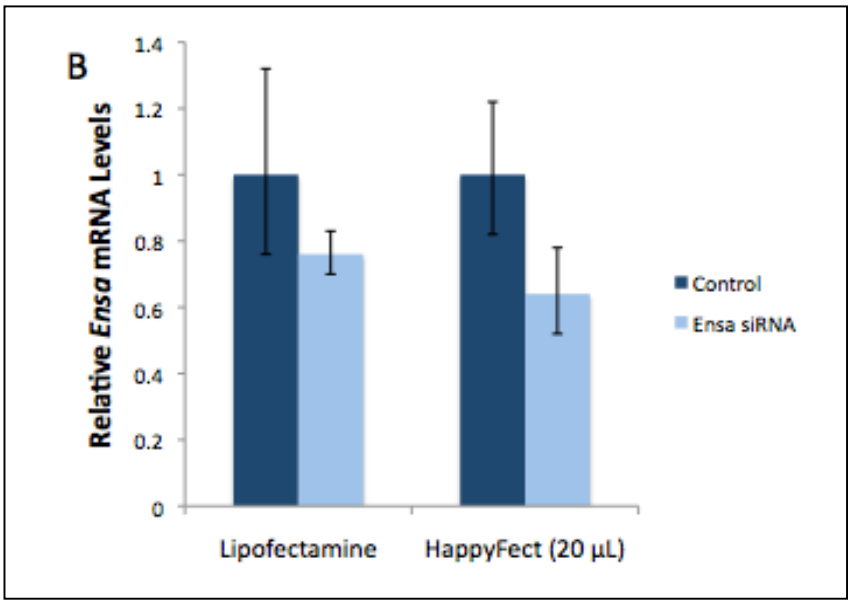

B
Relative Ensa mRNA Levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Lipofectamine
PEI (N/P 20)
Control
Ensa siRNA

Figure 8. A) Levels of *Ensa* transcript in NIH 3T3 cells, 44-48 hours following a transfection using PEI or PEGylated PEI as a delivery vehicle. The graph shows relative *Ensa* mRNA levels of cells transfected with a complex of PEI, PEGylated PEI, or Lipofectamine and *Ensa* siRNA (*Ensa* siRNA on x-axis) or PEI, PEGylated PEI, or Lipofectamine alone (control on x-axis). Complex of *Ensa* siRNA and polycations is formed at an N/P ratio of 10. **B)** Graph of relative *Ensa* mRNA levels of cells transfected with PEI or Lipofectamine and *Ensa* siRNA, or PEI or Lipofectamine alone; complexes of *Ensa* siRNA and PEI are formed at an N/P ratio of 20. For both experiments (A & B), Lipofectamine is used as a positive control. Relative *Ensa* mRNA levels are normalized to a reference gene, *Gapdh*. Error bars represent a pooled standard error of the variation in Ct values between triplicates for each primer set, as well as the variation between each experimental group (control and *Ensa* siRNA).


A
Relative Ensa mRNA Levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Ensa siRNA
Lipofectamine
HappyFect (10 µL)


B
Relative Ensa mRNA Levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Ensa siRNA
Lipofectamine
HappyFect (20 µL)

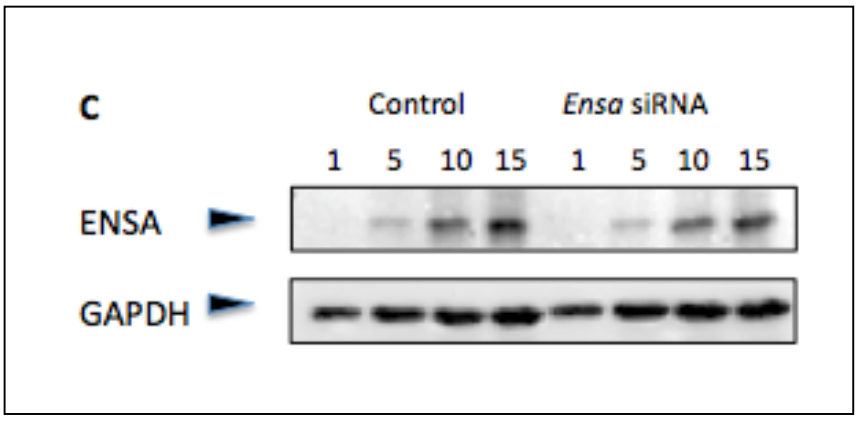

C
Control
Ensa siRNA
1 5 10 15 1 5 10 15
ENSA
GAPDH

Figure 9. A) Levels of *Ensa* transcript in NIH 3T3 cells, 44-48 hours following a transfection using HappyFect as a delivery vehicle. The graph shows relative *Ensa* mRNA levels of cells transfected with a complex of HappyFect or Lipofectamine and *Ensa* siRNA (*Ensa* siRNA on x-axis) or HappyFect or Lipofectamine alone (control on x-axis). 10 μL of HappyFect is used. **B)** Transcript levels of *Ensa* in NIH 3T3 cells, 44-48 hours following a transfection using 5 μL of HappyFect. For both experiments (A & B), Lipofectamine is used as a positive control. Relative *Ensa* mRNA levels are normalized to a reference gene, *Gapdh*. Error bars represent a pooled standard error of the variation in Ct values between triplicates for each primer set, as well as the variation between each experimental group (control and *Ensa* siRNA). **C)** Levels of ENSA protein in NIH 3T3 cells, 44-48 hours following a transfection using 10 μL of HappyFect as a delivery vehicle. Western blots using cell lysate from control cells (HappyFect only) or *Ensa* siRNA treated cells (HappyFect + *Ensa* siRNA) were prepared by probing for ENSA (top) and GAPDH (bottom) separately. Each blot shows protein bands for different amounts of cell lysate for each experimental group. The numbers above each blot represent the volume of cell lysate (μL) loaded in each well of the polyacrylamide gel. For knockdown calculations, ENSA protein levels were normalized to GAPDH levels in the same amount of cell lysate and the knockdown percentages were averaged.

Lipofectamine PEI (N/P 20)

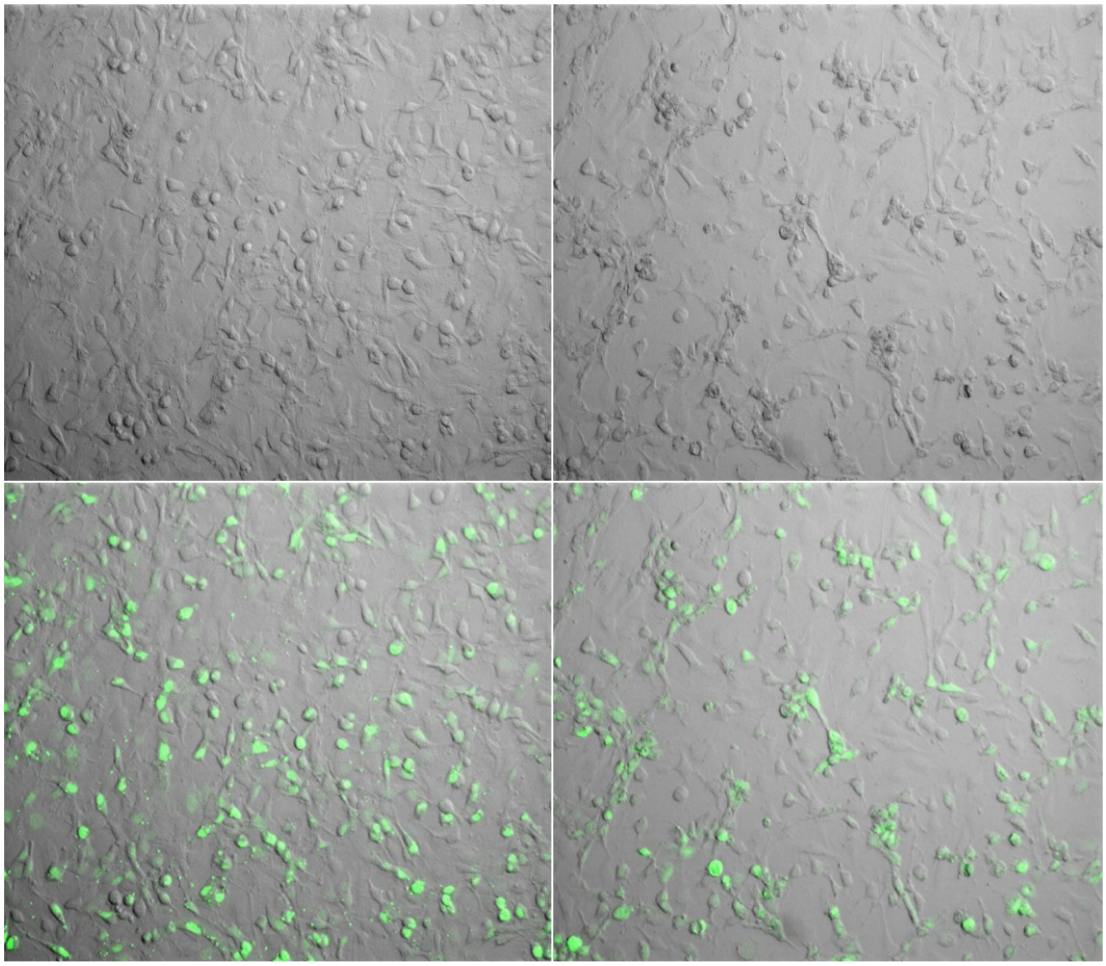


Figure 10. Uptake of the FITC labeled duplex using Lipofectamine or PEI as a delivery vehicle. NIH 3T3 cells were transfected with a complex of Lipofectamine and the FITC-duplex or a complex of PEI and the FITC-duplex. A complex of the duplex and PEI was formed at an N/P ratio of 20. Live cells were imaged on a Zeiss Axio Observer Z1. Images were taken with 10X resolution using differential interference contrast (DIC). Top two images are taken using transillumination (without the GFP filter). Bottom two images are the top images merged with images taken using the GFP filter.

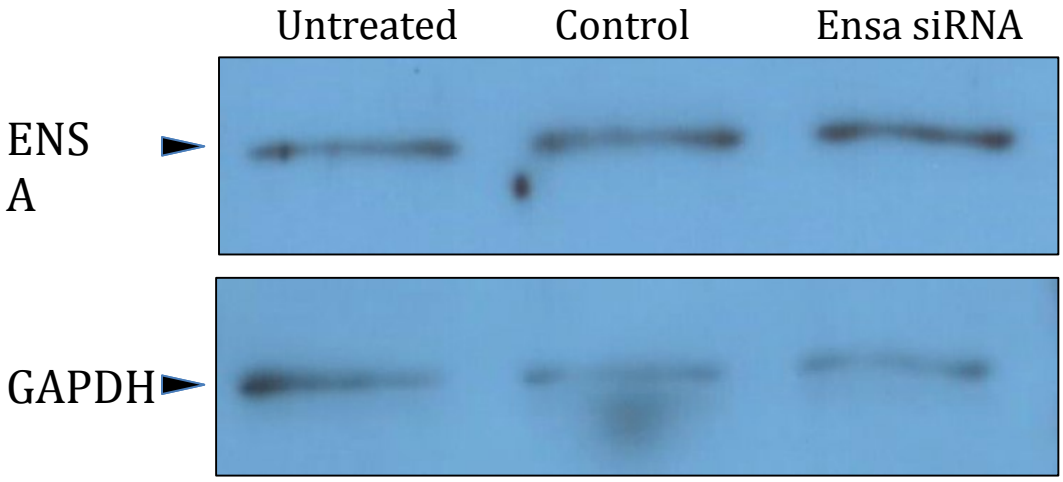

Untreated
Control
Ensa siRNA
ENS
A
GAPDH

Figure 11. Levels of ENSA protein in oocytes, 44-48 hours following treatment with HappyFect. Western blots using 20 control oocytes (HappyFect + Negative control siRNA) and 20 *Ensa* siRNA oocytes (HappyFect + *Ensa* siRNA) were prepared by probing for ENSA (top) and GAPDH (bottom) separately. To quantify knockdown, ENSA protein levels were normalized to GAPDH levels.

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Anita Ramachandran
237 S. Chester St., Baltimore, MD
508-505-1805
aramacha@jhsph.edu

EDUCATION

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD expected 2014
Sc.M., Biochemistry and Molecular Biology
First Year Literature Based Thesis: "MicroRNA Inhibition of a Tumor Suppressor: A Proposal for the Experimental Validation of the Determinants of MicroRNA repression"

Second Year Research Based Thesis: "Synthesis of a novel oocyte-specific delivery agent for siRNA mediated knockdown of alpha endosulfine"

McGill University, Montreal, QC 2011
B.A & Sc., Cell/Molecular Biology and International Development

Natick High School, Natick, MA 2007

RESEARCH EXPERIENCE

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD Present
Graduate Researcher, *Principal Investigators: Dr. Paul Miller, Dr. Janice Evans*

Design of a novel oocyte-specific gene delivery mechanism to inhibit expression of alpha endosulfine (ENSA)

- Utilizing quantitative RT-PCR and fluorescence microscopy to examine si-RNA mediated knockdown of ENSA in murine cells
- Synthesizing a polymer-based nanoparticle system
- Applying gel electrophoresis, size-exclusion chromatography, and dynamic light scattering methods to characterize the nanoparticle system
- Responsible for weekly presentations at lab meetings

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 2012-2013
Research Assistant, *Principal Investigator: Dr. Anthony Leung*

Investigation of the function of Poly ADP Ribose Polymerases (PARPs)

- Constructed a cDNA library of 17 PARP proteins using advanced molecular cloning techniques for application in future studies identifying the relationship between PARPs and miRNA-155
- Analyzed sequencing data using A Plasmid Editor (APE) software

Center for Inherited Disease Research, Baltimore, MD 2011-2012
Research Technologist, *Principal Investigator: Dr. Kim Doheny*

Interrogation of single nucleotide polymorphisms (SNPs) associated with human diseases

- Performed Illumina micro-array experiments to generate high throughput genotyping data
- Programmed liquid handling robots for DNA isolation and hybridization assays
- Responsible for daily lab maintenance and inventory

Johns Hopkins School of Medicine, Baltimore, MD 2011
Summer Research Assistant, *Principal Investigator: Dr. Sushant Kachhap*

Examination of the role of NDRG1 in DNA repair and prostate cancer metastasis

- Applied molecular cloning techniques, protein purification, SDS-PAGE, and Western Blots to investigate potential interactions between NDRG1 and other proteins

McGill University, Montreal, QC 2010-2011
Undergraduate Researcher*, Principal Investigator: Dr. Svetlana Komarova*

Behavioral evaluation of pain associated with bone metastasis

- Received animal training certificates from McGill University's Animal Resources Center for mouse handling
- Scored behavioral responses of mice using tests of mechanical sensitivity and motor impairment
- Aided with surgery of mice, including anesthetization and injection of breast carcinoma cell lines

McGill University, Montreal, QC 2009
Summer Undergraduate Researcher*, Principal Investigator: Dr. Hugo Zheng*

Mapping of plant cell-trafficking gene, cer11

- Learned to perform basic molecular biology techniques such as genomic DNA isolation, PCR and gel electrophoresis using Arabidopsis plants

RELATED PROFESSIONAL EXPERIENCE

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 2014
Teaching Assistant*, Molecular Endocrinology*

- Will lead review sessions to aid students with exam preparation
- Will grade online quizzes and exams

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 2013
Teaching Assistant*, Introduction to Reproductive Biology*

- Assisted in grading of final exams

Johns Hopkins School of Medicine, Baltimore, MD 2012
Volunteer at Community Science Day

- Prepared and taught an interactive lesson on basic genetic principles for 5th grade students at Moravia Park Elementary School

Kumon Learning Center, Natick, MA
2005-2007
Instructor

- Taught and mentored elementary and middle school students in Math and English
- Prepared, administered and corrected tests
- Organized a spelling bee competition for 50 students

CONFERENCE PRESENTATIONS

Johns Hopkins University, Baltimore, MD 2013
Delivered a poster presentation at the Institute for Biophysical Research Retreat
Title: "Synthesis and Characterization of an Oocyte-specific Nanoparticle Gene Delivery System"

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 2014
Will present at the annual Biochemistry and Molecular Biology colloquium

AWARDS AND RECOGNITION

- Williams College Book Award at Natick High School for high academic merit
- Ranked 9th in the country on National French Exam
- Silver Award on National Latin Exam

ADDITIONAL INFORMATION

- Proficient in STATA
- Competent in French, Tamil, and Hindi